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Structure-Function Relationships of Elements of the Bacteriophage f1 Genome

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STRUCTURE-FUNCTION RELATIONSHIPS OF ELEMENTS OF THE BACTERIOPHAGE f1 GENOME

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Abbreviations

Abbreviation	Definition
bp	Base pairs
BSA	Bovine serum albumin
cfu	Colony forming units
cp insert	f1 DNA fragment which contains the major coat protein gene transcription unit, bounded by EcoRI sites
cp vector	f1 phage cloning vector which contains a variant major coat protein allele in the normal position, and an EcoRI cloning site in the IG.
dNTP	dGTP, dATP, dCTP, or dTTP, or some combination of these
F	Frequently transcribed region of f1 genome
IF	Infrequently transcribed region of f1 genome
IG	f1 intergenic region located between genes IV and II
IPTG	Isopropyl-β-D-thiogalactoside, an inducer of the *lac* operon
kb	Kilobases
lacVIII tu	Hybrid transcription unit constructed by replacement of f1 nucleotides 1095-1177 on the wild-type cp insert with the E. coli *lacUV5* operator-promoter. The f1 major coat protein gene on the *lacVIII* tu is transcribed from the *lac* promoter and also from a new promoter fortuitously formed at the junction between the *lac* and f1 DNA sequences
MacGal	MacConkey galactose indicator plate
moi	Multiplicity of infection
PMSF	Phenyl methyl sulfonyl fluoride, a protease inhibitor
RF	Circular, double-stranded, replicative form DNA
RFI	Covalently closed, superhelical, circular, double-stranded replicative form DNA
SS	Circular, single-stranded, viral DNA
TCA	Trichloroacetic acid
XG	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Abstract

Expression of the ten genes on the small filamentous phage f1 genome must be closely regulated during the infection cycle, since this nonlytic coliphage lives in delicate balance with its host. The sequence and context requirements of specific regions involved in f1 mRNA metabolism were investigated by: 1) relocation of an intact transcription unit on the phage genome; 2) construction of a hybrid E. coli-f1 transcription unit which could be studied in a phage or plasmid context; 3) identification and characterization of a rho-dependent transcription termination signal in f1 by cloning it between the promoter and gene of an E. coli transcription unit, and by studies of its function in its usual phage context.

Expression of the f1 major coat (gene VIII) protein was studied in vivo in cells infected with variant f1 phage whose genomes had been restructured in vitro. This gene with its most proximal promoter and terminator was found to behave as an independent transcription unit. Coat protein gene expression was unaffected by transposition of the gene to the phage's large intergenic region, and was independent of orientation, but was dependent upon its own intact promoter.

Gene VIII was placed under control of the E. coli lac promoter and the RNA from the new transcription unit was characterized. Coat protein was expressed from a hybrid mRNA initiated at the lac promoter. This mRNA, which contained the 5' 36 nucleotides of the lac operon mRNA at its 5'end, attached to the entire gene VIII mRNA sequence, was unstable (half life ~1.5 min), as is the E. coli lac operon mRNA. In contrast, authentic f1 gene VIII mRNA is extremely stable (half life ~10 min).

The f1 large intergenic region was shown to encode a rho-dependent transcription termination signal. The minimal sequence required for terminator function in a heterologous plasmid system encompasses about 100 nucleotides. Like most known rho-dependent terminators, the signal contains a region of dyad symmetry. It differs from previously characterized rho-dependent terminators, in that the sequence at the termination site is G-C rather than A-T rich. In a rho mutant host, f1 tran-

scripts pass through the normal termination site, and stop downstream within a region of high potential secondary structure near the f1 origin of DNA replication.

A method is described for the efficient construction in vitro of recombinant DNA molecules from fragments produced by cleavage with the restriction endonuclease HgaI. The method relies upon the unique properties of HgaI and is applicable to any viral or plasmid DNA that contains several HgaI recognition sites. Using f1 DNA, it is shown that only HgaI fragments that were originally adjacent on the genome can anneal, that infectious molecules are reassembled with high efficiency from a mixture of fragments, and that recombinant genomes can be easily constructed from parental DNAs containing genetic markers which map in different HgaI fragments.

Chapter 1
Introduction

The filamentous coliphages provide a very simple model system for the analysis of genome organization and gene regulation. The entire nucleotide sequence of their DNA has been determined (Beck et al., 1978; van Wezenbeck et al., 1980; Hill and Petersen, 1982), and most of the coding regions have been correlated both genetically and physically with the gene products. Regulatory sequences for DNA replication and RNA transcription have been identified in vitro and in vivo, but the significance of the phages' genome organization and the detailed mechanisms governing regulation of expression of the products are as yet incompletely understood. The compact organization of the genome necessitates that DNA sequences be used for more than one function. The phages' delicate balance with their host cells suggests that the regulatory system is finely tuned. This thesis examines several regulatory features of RNA synthesis and turnover in the filamentous phage f1. The approach taken was to remove regulatory regions from their normal environment so they could be studied in isolation or in a new environment.

Filamentous Phage Life Cycle

The filamentous phages f1, fd, and M13 are nearly identical in sequence and structure and can be considered the same organism (described in detail by Denhardt et al., 1978). They infect only E. coli carrying the F (or sex) factor, and hence are known as male-specific bacteriophages. Infection is mediated by attachment to the end of the F-pilus (Caro and Schnös, 1966), a filamentous projection from the cell surface encoded by the F-factor. Retraction of the pilus brings the virion to the cell surface, and the single-stranded DNA enters the bacterium (Griffith et al., 1981), while the coat protein molecules dissociate from the DNA and remain in the bacterial membrane where they can be re-used for the encapsidation of progeny phage (Smilowitz, 1974). However, phage DNA can be introduced by transfection into F^- (female) cells, in which the complete phage life cycle is carried out and virions infectious for F^+ cells are released.

Filamentous phage establish a persistent infection in which the host cells continue to grow and divide, although at a reduced rate. Phage-specific replication, transcription, and translation proceed, and mature virions are continuously assembled at and then extruded through the cell membrane. Each virion is composed of a circular 6407-nucleotide single-stranded DNA molecule encapsidated by about 2700 monomers of the major coat protein, the product of gene VIII, and 5-10 monomers each of several minor coat protein molecules, the products of genes III, VI, VII, and IX (Goldsmith and Konigsberg, 1977; Lin et al., 1980; Grant et al., 1981; Simons et al., 1981). Virions resemble long filaments since the viral DNA is stretched out within the tubular core of gene VIII protein monomers (Marvin, 1978), with the minor proteins localized at one tip (gene III and VI proteins) or the other (gene VII and IX proteins) (Rossomondo and Zinder, 1968; Goldsmith and Konigsberg, 1977; Grant et al., 1981; Simons et al., 1981). The latter tip probably is extruded through the membrane first and the former tip seems to exit last (Pratt et al., 1969). Interaction of intracellular viral single-stranded DNA molecules complexed with phage single-stranded DNA binding protein (gene V protein) (Pratt and Erdahl, 1968; Alberts and Frey, 1972), with gene VIII coat protein molecules located in the cell membrane leads to the replacement of gene V protein by gene VIII protein as the virions are extruded. The primary translation product of gene VIII is a 73-amino acid precursor molecule which is processed at the cell membrane to the 50-amino acid mature form found in virions (Nakashima and Konigsberg, 1974; Pieczenik et al., 1974; Sugimoto et al., 1977; Chang et al., 1978). This mature form consists of an acidic N-terminal domain protruding outside from the membrane and later from the virion, a hydrophobic interior domain, and a basic C-terminal domain thought to interact with the viral DNA.

Genome Organization and Expression

Upon infection of the bacterium, the single-stranded circular phage genome is converted to the double-stranded replicative form (RF), which serves as the template for transcription of the phage genes and for further replication. DNA replication requires the products of genes

II and V: gene II protein creates a site-specific nick at the origin of viral (+) strand replication (Meyer and Geider, 1979; Dotto et al., 1981a), and gene V protein binds to viral single strands to prevent their conversion to RF (Mazur and Model, 1973; Mazur and Zinder, 1975). In the absence of gene V protein, complementary (-) strand DNA synthesis initiates via an RNA primer (ori-RNA) synthesized by E. coli RNA polymerase at the (-) strand origin (Geider et al., 1978), which is located close to the (+) strand origin. Thus, viral strand replication takes place by a rolling circle mechanism, after which the newly synthesized (+) strands are either doubled up to give RF (early in infection) or sequestered by binding protein and used for progeny phage production (late in infection) (Horiuchi and Zinder, 1976). However, there is no true early/late switch and so both processes occur to some extent both early and late.

Figure 1.1 shows a map of the f1 genome, opened between genes VIII and III. The DNA replication protein genes II (X is a carboxy terminal subset of II; Yen and Webster, 1981) and V are adjacent, followed by the capsid protein genes VII, IX, VIII, III, and VI in that order. The remaining two genes, I and IV, are of unknown function but are required for morphogenesis. The 505 bp intergenic region (IG) between genes IV and II contains the origins of (+) and (-) strand DNA replication (Tabak et al., 1974; Horiuchi and Zinder, 1976; Suggs and Ray, 1977) and a DNA packaging signal for morphogenesis (Dotto et al., 1981b). Foreign DNA can be inserted at certain locations in the IG and the resultant chimeric DNA molecules propagated as helper independent phage (Messing et al., 1977; Herrmann et al., 1978; Boeke et al., 1979).

The genome can be divided into three regions with regard to transcription. The infrequently transcribed (IF) region covers genes III, VI, I and IV. The frequently transcribed (F) region covers genes II, V, VII, IX, and VIII. The IG, which separates the IF and F regions, appears not to be transcribed at a detectable level (Smits et al., 1978, 1980; Cashman and Webster, 1979; Cashman et al., 1980; LaFarina and Model, 1983). In vitro studies have established that all genes but VII and IX are preceded by a promoter sequence, but there is no promoter im-

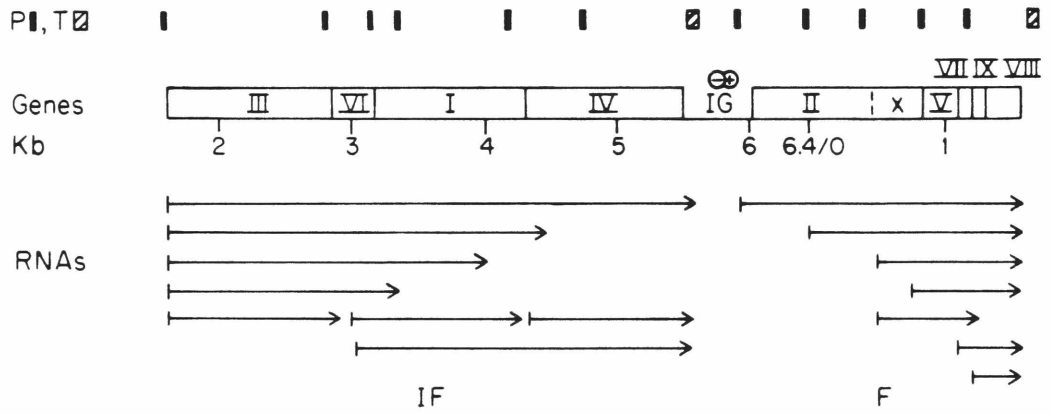


Figure 1.1 f1 genome organization and transcription map. The circular f1 genome is shown opened between genes VIII and III. Genes are denoted by Roman numerals, nucleotide positions in kb are from the unique HincII site. IG is the intergenic region which contains the origins of (-) and (+) strand DNA synthesis. Promoter sites are marked ▮, transcription termination sites ▨ (Beck and Zink, 1981). The infrequently transcribed (IF) and frequently transcribed (F) regions are indicated below the genetic map along with the in vivo RNAs characterized by La Farina and Model (1983).

mediately preceding the IG. The G-start promoters of the F region are in general stronger than the A-start promoters of the IF region, the strongest being those preceding genes X, II, and VIII (Seeburg and Schaller, 1975; Okamoto et al., 1975; Edens et al., 1976; Seeberg et al., 1977). Transcription proceeds in one direction as shown in Figure 1.1; therefore, all mRNAs have the same polarity as the viral strand. In vivo, termination occurs at specific sites at the ends of genes VIII (rho-independent) (Edens et al., 1975; Sugimoto et al., 1977; Rivera et al., 1978; Cashman et al., 1980), and IV (rho-dependent) (as suggested by Smits et al., 1978,1980; Chapter 6, this thesis). A series of 3'coterminal RNAs ranging in size from 370 to 2000 nucleotides have been mapped for the F region. The smallest RNA, which codes for the major coat (gene VIII) protein, is by far the most abundant, accounting for up to 2% of the total RNA in infected cells (Rivera et al., 1978; Cashman and Webster, 1979; Cashman et al., 1980; Smits et al., 1980; LaFarina and Model, 1983). The RNAs from the IF region seem to share common sequences at their 5'ends (Smits et al., 1980; LaFarina and Model, 1983).

Expression of the f1 genes must be regulated in some manner, since their proteins are produced in vastly different amounts. The major coat protein and the single-stranded DNA binding protein are the most abundant, while the others are difficult to detect in vivo. Amber mutants in all genes except II kill non-permissive hosts (Pratt et al., 1966, 1969), presumably by perturbation of the normal delicate balance between phage gene expression and host cell metabolism (gene II mutants do not synthesize DNA beyond the parental RF, so the gene products are barely expressed). Seeking to understand better the significance of f1's sequence and genome organization, I addressed several specific questions. Is the normal location of gene VIII at the end of the F region necessary for its expression at high levels? What role do proximal sequences play in promoter function and mRNA stability? What would be the effect of a high rate of transcription within the IG, a region that is normally not transcribed? Does the rho-dependent transcription termination site, inferred from the absence of RNA complementary to IG sequences (Smits et al., 1980; LaFarina and Model, 1983), really function as such in vivo,

and can its important features be characterized? These questions were studied by molecular dissection and rearrangement, so that regulatory element and gene function in vivo could be tested in contexts other than those of a normal phage infection.

Chapter 2
Materials and Methods

Purification of restriction endonuclease HgaI

HgaI, from Haemophilus gallinarum ATCC 14385, was purified by a modification of the procedure of P. A. Myers and R. J. Roberts (personal communication). Cells were grown in brain/heart infusion (37 g/l; Difco) supplemented with 10 ug/ml hemin and 2 ug/ml NAD after autoclaving, harvested by centrifugation, sonicated, and the nucleic acids precipitated with streptomycin sulfate. Following ammonium sulfate precipitation, the protein fraction was chromatographed on phosphocellulose (Whatman P11) and then on DEAE-cellulose (Whatman DE52), using linear KCl gradients in PC buffer (0.01 M KH_2PO_4 (pH 7.4)/0.01 M β-mercaptoethanol/0.1 mM EDTA/10% glycerol). Peak fractions were pooled and concentrated by dialysis against PC buffer containing 50% glycerol. Bovine serum albumin (BSA; Pentex) was added to 200 ug/ml, and the enzyme was stored at -20°.

Later purification of HgaI was done by an alternate and better procedure of Myers and Roberts. All steps were completed as rapidly as possible after sonication of the cells, since HgaI activity is rather unstable. Fourteen grams of cells grown to OD_{700} = 1.5, harvested as above, and stored at -20° were thawed and resuspended in 23 ml of 0.01 M KH_2PO_4 (pH 7.4)/0.01 M β-mercaptoethanol, sonicated at 4°, and centrifuged at 4° for 2 hr at 100,000 g (SW40 rotor, 36,000 rpm). The supernatant was diluted 1:1 with PC buffer containing 70 ug/ml PMSF (phenyl methyl sulfonyl fluoride, a protease inhibitor) and dialyzed against PC buffer/PMSF at 4° overnight. The dialysate was applied to a 2 x 15 cm Whatman DE52 column in PC buffer/PMSF and block-eluted with 120 ml of 0.1 M KCl/PC buffer/PMSF. HgaI elutes at 0.05 M KCl on this column. The peak fractions were diluted 1:1 with PC buffer/PMSF, applied without dialysis to a 0.9 x 25 cm Whatman P11 column in 0.05 M KCl/PC buffer/PMSF, and eluted with an 80 ml linear gradient of 0.05-1.0 M KCl/PC buffer/PMSF. HgaI elutes at 0.68 M KCl on this column. The peak fractions were dialyzed against PC buffer without PMSF, applied to a 0.9

x 15 cm DE52 column, and block-eluted with 0.08 M KCl/PC buffer. Peak fractions were pooled, concentrated by dialysis against 0.01 M Tris-HCl (pH 7.4)/0.01 M β-mercaptoethanol/0.1 mM EDTA/50% glycerol, and stored frozen in liquid nitrogen. Aliquots for use were thawed, and after the addition of 200 ug/ml BSA, were stored at -20°. HgaI activity was stable through liquid nitrogen storage and initial thawing, but deteriorated over several months of storage at -20°.

Digestion and ligation conditions for in vitro recombination promoted by HgaI

Digestions were performed with 4 ul of HgaI per ug DNA at a DNA concentration of 0.06 ug/ul in 7 mM Tris-HCl (pH 7.4)/7 mM $MgCl_2$ at 37°, and terminated by heating at 65° for 10 min. Ligations with bacteriophage T4 DNA ligase (a generous gift from Dr. C. Yehle) were carried out in 20 mM Tris-HCl (pH 7.4)/7 mM $MgCl_2$/0.1 mM EDTA/50 ug/ml BSA/ 100 uM ATP/1.4 mM β-mercaptoethanol at 12° for 20 hr, and terminated by heating as above.

Phage DNA synthesis measurement

Cells were grown at 37° in D0 medium [0.2 g $MgSO_4 \cdot 7H_2O$/ 2 g citric acid/10 g K_2HPO_4/3.5 g $NaNH_4HPO_4 \cdot 4H_2O$ per l (Vogel and Bonner, 1956); supplemented with 1-2 mM amino acids (minus phenylalanine), 0.5% glucose, and 5 ug/ml thiamine] from a 1:30 dilution of an overnight culture, for 1.75 hr. Cultures were prelabeled for more than one generation (generation time = 40 min in D0 medium at 37°) by the addition of thymidine to 4 ug/ml, deoxyadenosine to 200 ug/ml, and [^{3}H-methyl]-thymidine (20.0 Ci/mmole; New England Nuclear) to 10 uCi/ml of culture. Under these conditions incorporation of label increased linearly for at least 2 hr. When the culture reached a density of 4 x 10^8 cfu/ml (approximately 1 hr after label addition), phage were added at moi 50. Ten min later a portion of the culture was treated with anti-f1 antiserum at K=3 for 10 min at 37° and plated for infective centers and surviving cells. In all cultures about 60% of the cells were infected. At 30 min postinfection the cultures were harvested, lysed, and the DNA sedimented through neutral 5-20% sucrose gradients in 1 M NaCl, which were frac-

tionated and counted to determine the levels of phage RF and single-stranded DNA (Mazur and Zinder, 1975).

Phage coat protein synthesis measurement

Cells were grown in DO medium lacking phenylalanine as above to 4 x 10^8 cfu/ml and infected at moi 50. At 30 min postinfection 1 uCi ^{14}C-phenylalanine (460 mCi/mmole; Schwarz-Mann) was added to each 200 ul infected culture and uninfected control culture for a labeling period of 5 min at 37°. The cultures were then placed on ice and precipitated by the addition of 2 mls of 5% TCA. After centrifugation, the pellets were resuspended in 100 ul of water, frozen in a dry-ice ethanol bath, and dessicated over NaOH pellets in a vacuum for at least 5 hrs. The proteins were resuspended in 100 ul of sample buffer (LaFarina and Model, 1978), boiled 3 min, and 20 ul samples were electrophoresed on SDS/urea/polyacrylamide gels containing an exponential 22-15% gradient of acrylamide as described by Chang et al. (1978). The gels were fixed for 10 min in 50% methanol/7% acetic acid and then immersed in 10 vol of 1 M Na-salicylate (reagent grade) for 30 min (Chamberlain, 1979). The gels were dried on Whatman 3MM paper and exposed to Dupont Cronex II film at -70°. The bands corresponding to f1 gene VIII protein were cut out from the dried gels and counted in POPOP/PPO/toluene in a scintillation counter.

Alternatively, SDS/urea gels containing 23% acrylamide/0.088% bisacrylamide were used, followed by autoradiography (Boeke et al., 1980). Where indicated, the labeled cultures were separated into phage (supernatant) and cellular (pellet) fractions by centrifugation before TCA precipitation of proteins. Phage and cellular samples were electrophoresed in separate gel lanes. Wild type f1 and R235-type coat protein peak areas determined by microdensitometer scanning within a single gel lane were compared to determine, separately for the virions and intracellularly, the relative amount of each coat protein type in a given partial diploid phage strain.

Phage gel system

The gels consisted of 2% agarose in 0.37 M Tris-glycine (pH 9.5)

TABLE 2.1

E. coli Strain Derivations

Strain	Genotype	Source or reference
K37	HfrC(λ) supD	Lyons and Zinder, 1972
K38	HfrC(λ) sup^+	Lyons and Zinder, 1972
K484	KL16 HfrPO45 thi1 rel1 λ^-	B. Bachmann
K508	K37.pMB4 (see Table 2.2)	Boeke, 1982
K535	K38.pBR322	Transformation with pBR322; Bolivar et al., 1977
K551	JC10240 HfrPO45 recA56 srl300::Tn10 thr300 ilv318 rpSE300	A. J. Clark
K561	K38 HfrC(λ) lac^{iQ}	Boeke, 1982
K701	C600 $galE^+T^+K^-$ lac^- thr^- leu^-	McKenney et al., 1981
K702	N100 $galE^+T^+K^-$ pro^- $recA^-$	McKenney et al., 1981
K732	RK4349 F^- pro3 entA403 his218 ilvC7 metB1 DE6(∇lac) xyl^- rpsL107 λ^- supE44 metE163::Tn10	B. Bachmann
K734	HD173 F^- thr33 trpE9829 tyrA15 thyA707 argH λ^- ilv683 nitA702ts	B. Bachmann; Inoko et al, 1977
K760	K734 ilv683 nitA702ts metE163::Tn10	P1 transduction from K732; M. Russel
K782	K701 $galE^+T^+K^-$ lac^- thr^- leu^- ilv683 nitA702ts metE163::Tn10	P1 transduction from K760
K791	K701 $galE^+T^+K^-$ lac^- thr^- leu^- recA56 srl300::Tn10	P1 transduction from K551
K792	K782 $galE^+T^+K^-$ lac^- thr^- leu^- ilv683 nitA702ts	Spontaneous reversion
K793	K792 $galE^+T^+K^-$ lac^- thr^- leu^- ilv683 nitA702ts recA56 srl300::Tn10	P1 transduction from K551
K819	K38 HfrC(λ) ilv683 nitA702ts metE163::Tn10	P1 transduction from K760; M. Russel
K829	BL214 HfrPO45 uraP119 λ^- rnc105	Studier, 1975
K830	SA500 ilv^- his^- Tn10 near nusA1 (30% linkage)	M. Gottesman
K831	K38 HfrC(λ) Tn10 nusA1	P1 transduction from K830
K836	K38·pEG25 (rho^+ allele cloned on pBR322)	Transformation with pEG25; Gulleta, E. and Adhya, S. manuscript in preparation
K837	K819·pEG25	Transformation with pEG25
K838	K819·pBR322	Transformation with pBR322

TABLE 2.2

Plasmid Strains

Plasmid	Relevant characteristics[a,b]	fl Insert name	fl insert orientation[c]	Reference
pBR322	Cloning vector			Bolivar et al. 1977
pEG25	*rho*$^+$ allele cloned in pBR322			Gulleta, E. and Adhya, S. in prep.
pKG100	*Pgal*-multiple cloning sites-*galK*			McKenney et al.1981
pKG1900	*Pgal*-SmaI site-*galK*			"
pKG1900B	*Pgal*-BamHI site-*galK*			footnote[d]
pMB4	r_{RI}^+, m_{RI}^+			Betlach et al. 1976
pJB1	*lacOP*-1178-1926 (IX,VIII,IIIF)	*lacOP*-fl ThaI/HpaIIC	-	Boeke et al. 1982
pJB2	"	"	+	"
pJB81	1096-1926-*lacOP* (VII,IX,VIII$_{am}$,IIIF)	R267 HpaIIC-*lacOP*	+	Boeke 1982
pJB84	1178-1926-*lacOP* (IX,VIII,IIIF)	R274 ThaI/HpaIIC-*lacOP*	+	"
pJB85	"	"	-	"
pPM1	5148-5616/pKG1900B	HgaI/HpaII 469	+	Chapter 6[e]
pPM2	"	"	-	"
pPM3	5416-5616/pKG100	HaeIII/HaeII 201	+	"
pPM4	"	"	-	"
pPM5	5488-5616/pKG100	RsaI/HpaII 129	+	"
pPM6	"	"	-	"
pPM7	5535-5616/pKG100	ThaI/HpaII 82	+	"
pPM8	"	"	-	"
pPM9	5416-5563 or 5571/pKG100	HaeIII/HaeII 150	+	"
pPM10	"	"	-	"
pPM11	5416-5579/pKG100	HaeIII/MboII 167	+	"
pPM12	"	"	-	"

[a] All plasmids listed are derived from pBR322, and all confer ampicillin resistance upon the host cell.

[b] f1 sequences cloned are indicated by nucleotide position, with genes and gene fragments (F) contained in parentheses.

[c] For pJB plasmids (f1 sequences cloned in pBR322), + orientation is defined as f1 viral strand transcripts proceeding towards pBR322 tetracycline resistance genes.

For pPM plasmids (f1 sequences cloned in pKG vectors), + orientation is defined as f1 viral strand transcripts proceeding towards galK gene.

[d] The pKG1900 SmaI site was converted to a BamHI site by insertion of a BamHI linker.

[e] f1 fragment HgaI/HpaII 469 with BamHI linkers, and f1 fragments HaeIII/HpaII 201, ThaI/HpaII 82, and HaeIII/HaeII 150 with HindIII linkers were kindly provided by G. P. Dotto. Fragments RsaI/HpaII 129 and HaeIII/MboII 167 were derived by cleavage of HaeIII/HpaII 201 with RsaI or MboII, followed by the attachment of HindIII linkers. Fragments were cloned in the BamH1 site of pKG1900B or the HindIII site of pKG100 as indicated.

TABLE 2.3

Phage Strains

Strain	Genotype	Orientation	Construction	Reference
f1	wild type			Loeb 1960; Zinder et al. 1963
am8H1	VIIIms53,VIIIaml			Pratt et al. 1969
R16	Vaml6			Lyons & Zinder 1972
R24	IVaml2,IIam24			"
R30	Vaml3,IIam30			"
R79	IVaml2,Iam79			"
R199	EcoRI site at 5726			Boeke et al. 1979
R211	VIIIms53,VIIIaml,EcoRI site at 5726		a	Chapter 3
R212	R211/cp insert f1	+	b	Chapter 4
R214	IVaml2,EcoRI site at 5726		a	Chapter 3
R215	Vaml6,EcoRI site at 5726		a	"
R229	EcoRI site at 5616			Boeke 1981
R235	VIIIms235			Braunitzer et al. 1970
R240	VIIIms53,VIIIaml,VIIIms240			Boeke et al. 1980
R252	VIIIms53,VIIIaml,EcoRI site at 5616		a	Chapter 4
R253	R252/cp insert f1	+	b	"
R254	"	-	b	"
R258	VIIIms53			Pratt et al. 1969
R265	VIIIms265,VIIIaml,EcoRI site at 5616		d	Chapter 4
R266	R265/cp insert R265 lacOP	+	c	"
R267	R252/cp insert am8H1 lacOP	-	c	"
R268	R252/cp insert VIIIms268,VIIIaml lacOP	+	c	"
R269	R252/cp insert f1 lacOP	-	b	"
R270	VIIIms235,EcoRI site at 5616		a	"
R271	R270/cp insert f1 lacOP	+	b	"
R272	"	-	b	"
R273	R270/cp insert (Δ-35 region) lacOP	+	b	"
R274	"	-	b	"
R275	R270/lacOP(Δ-35 region)cp insert	+	b	Chapters 4,5
R276	"	-	b	"
R277	VIIIms53,EcoRI site at 5616		a	Chapter 4
R278	R277/cp insert f1 lacOP	+	b	"
R279	R252/cp insert(Δ 5'end) lacOP	-	b	"
R280	VIIIms53,VIIIaml,VIIIms240,EcoRI site at 5616		a	"
R281	R280/cp insert f1 lacOP	+	b	"
R282	"	-	b	"

[a] HgaI-promoted recombination in vitro.
[b] Gene VIII partial diploid constructed by in vitro cutting and ligation.
[c] UV mutagenesis in vitro of the cp insert from R269, followed by recloning in R252.
[d] From R266 by excision of the cp insert with EcoRI, followed by religation of the vector molecule in vitro.

and were run in the same buffer at 8 V/cm (Beaudoin, 1970). The gels were then immersed for 45 min in 0.2 N NaOH to disassemble the virions in situ, rinsed briefly with water, and then immersed in 0.5 M Tris-HCl (pH 7.4) for 15 min to neutralize the NaOH. Ethidium bromide was added to 0.5 ug/ml for 30 min to stain the viral DNA, and the gels were photographed under UV light (Nelson et al., 1981).

Amino acid analysis

Amino acid analysis of protein from purified virions was performed on a D-500 amino acid analyzer by Dr. Stanford Moore, for whose interest in, and assistance with, my project I am extremely grateful.

General recombinant DNA techniques

Plasmid and phage RF were isolated as described (Boeke, 1982), and when necessary, further purified by isopycnic centrifugation in CsCl/ethidium bromide (Model and Zinder, 1974). Restriction mapping of recombinant plasmid and phage RF was performed as described by Horiuchi et al. (1975) using 2.5-7.5% or 2.5-10% acrylamide gradient gels or vertical agarose gels as described, or horizontal agarose gels run in TBE buffer (0.089 M Trizma base/0.089 M boric acid/2.8 mM EDTA, pH ~8.3). DNA fragments were purified by electrophoresis on acrylamide gradient, or on 4% or 8% acrylamide slab gels. Whenever possible, preparative amounts of fragments were visualized by placing the unstained gel over a fluorescent thin layer plate and shining UV light from above, in order to avoid the use of ethidium bromide staining. Fragments were eluted from gels by soaking or electrophoresis according to Boeke (1982). Restriction enzyme linearized vector DNAs (20 ug) were dephosphorylated with bacterial alkaline phosphatase (6 ug; Worthington) or calf intestine alkaline phosphatase (3 ug; Boehringer-Mannheim) at 37° for 1 hr, terminated by phenol extraction for the bacterial or heating at 65° for the calf enzyme, in order to prevent intramolecular ligation of vector molecules (Ullrich et al., 1977). Restriction site linkers were constructed by the biological linker technique of Boeke et al. (1979) or obtained from Collaborative Research. Ligation of blunt or 5'protruding DNA ends with bacteriophage T4 DNA ligase (generously sup-

plied by J. Boeke) was performed as described for HgaI-promoted recombination, but at 4°.

UV mutagenesis in vitro

The 930 bp EcoRI restriction fragment "cpb insert" (Chapter 4) was irradiated at 70 cm from a UV germicidal lamp for 10 min (1 lethal hit per 580 bp f1 RF) and then ligated to unirradiated vector RF. The ligated DNA was used to transfect unirradiated, $CaCl_2$-treated recA$^+$ E. coli cells.

Transfection, transformation, and transduction

Transfection with phage or transformation with plasmid RF was by the $CaCl_2$ technique (Mandel and Higa, 1970). Cells were grown in tryptone broth (10 g tryptone/8 g NaCl/1.0 g yeast extract/1.0 g dextrose/1.5 ml 1 N NaOH per 1 water) to OD_{660} = 0.6, centrifuged, resuspended in 0.5 vol 50 mM $CaCl_2$, and incubated on ice for 15-90 min. The cells were then recentrifuged, resuspended in 0.05 vol 50 mM $CaCl_2$, and allowed to settle on ice for 3 hr or longer. Resuspended cells (0.1-0.3 ml) were added to DNA solution in 50 ul DB (0.01 M Tris-HCl (pH 7.4)/0.01 M NaCl/0.2 mM EDTA), incubated on ice 10 min, at 37° 2.5 min, and then plated with 2.5 ml soft agar on tryptone agar plates (transfection) or diluted 10-fold with tryptone broth and allowed to grow at 37° for 30-60 min prior to plating on tryptone plates containing 20 ug/ml ampicillin (transformation). The dye 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XG; Bachem) was incorporated in tryptone plates at 40 mg/l as indicator for β-galactosidase activity (Miller, 1972), when screening for the presence of the lacOP DNA fragment.

P1 transduction was performed according to Miller (1972).

DNA sequencing

DNA was sequenced by the dideoxy terminator method of Sanger et al. (1977) or the chemical degradation method of Maxam and Gilbert (1980) with electrophoresis on ultrathin gels (Sanger and Coulson, 1978). Template phage single-stranded DNA for dideoxy sequencing was purified as described by Boeke (1982).

RNA labeling, extraction, and gel analysis

Cells were grown at 37° in peptone (10 g peptone/5 g NaCl/1 g dextrose per 1 water), plus 20 ug/ml ampicillin for plasmid-harboring strains, and 10^{-3} M isopropyl-β-D-thiogalactoside (IPTG; Sigma) as inducer when maximal transcription from lacOP was desired (Miller, 1972). Log phase cultures at 2 x 10^8 cfu/ml were infected with phage at moi 50-100 and 15 min later ^{32}P-phosphate was added to a final concentration of 50 uCi/ml; plasmid-harboring strains were likewise labeled at 2 x 10^8 cfu/ml. Labeling was conducted at 37° for 30 min, and the RNA extracted immediately as described below. For analysis of mRNA decay, rifampicin was added at the end of the labeling period to 200 ug/ml final concentration (0.11 vol of a fresh 2 mg/ml solution in 0.01 N NaOH; Sigma), and samples were removed just prior to, 2 min after, and 10 min after rifampicin addition. Under these conditions, incorporation of ^{3}H-uridine into TCA-precipitable material was reduced by 80% within 30 sec of rifampicin addition.

Labeled cultures (0.5 ml) were transferred to eppendorf tubes in an ice-water bath, and then spun in a microfuge 2 min at 4°. The pellets were rapidly resuspended in 50 ul cold RNA buffer (0.01 M Tris-HCl (pH 7.4)/0.01 M NaCl/5 mM $MgCl_2$), 5 ul of 10% SDS added, and the tubes placed at 65° for 3 min to lyse the cells. The lysates were diluted with 0.45 ml of 0.1 M Na-acetate (pH 5.2), 0.5 ml of phenol (equilibrated with 0.01 M Tris-HCl (pH 8)/1 mM EDTA; AnalaR) added, and the tubes vortexed 3 min and then spun 5 min. The aqueous phases were transferred to clean tubes, leaving behind material at the phenol/water interface (much of the DNA partitions here at pH 5.2), and phenol extracted twice more. The RNA was precipitated by the addition of Na-acetate to a final concentration of 0.2 M, plus 3 vol absolute ethanol, collected by centrifugation, dried in vacuo, resuspended in 50 ul sterile water, and stored at -20°.

Vertical slab gels were 4% acrylamide/0.2% bisacrylamide in TBE buffer. Samples contained 1-2 ul RNA (~1 x 10^5 cpm) in 10 ul of loading buffer [0.025 M Tris-acetate (pH 6.8)/1 mM EDTA/0.5% β-

mercaptoethanol/0.5% SDS/ 5% glycerol/0.03% bromophenol blue/50 ug/ml tRNA] and were heated at 100° for 60 sec prior to loading. Electrophoresis was at 80 V until the bromophenol blue dye had just run off the gel's bottom edge. The gels were dried on Whatman 3MM paper and autoradiographed at room temperature.

Preparation of 3'end-labeled DNA probes

Restriction fragments were labeled using all α-^{32}P-dNTPS which were incorporable opposite the single-stranded protruding 5'ends. Reactions containing 4 pmoles fragment (15 ug f1 RF-equivalents) and 50 uCi of each α-^{32}P-dNTP (3000 Ci/mmole; Amersham) in Hin buffer [6 mM Tris-HCl (pH 7.5)/6 mM NaCl/6 mM $MgCl_2$/6 mM DTT], total volume 39 ul, were incubated with the Klenow fragment of DNA polymerase I [1 ul of a 6-fold dilution in 0.1 M KPO_4 (pH 7)/50% glycerol; Boehringer-Mannheim] for 30 min at room temperature. The reactions were chased by the addition of 1 ul of each of the 4 unlabeled dNTPs from 0.5 mM stock solutions, followed by incubation for 30 min at room temperature. Fragments were phenol extracted and ethanol precipitated if cleavage by a second enzyme was required to remove undesired sequences, or label at one end prior to DNA sequencing. Labeled fragments were repurified by electrophoresis on polyacrylamide gels, elution, and ethanol precipitation, resuspended in sterile water, and stored at -20°. The specific activity of the probes was usually about 5×10^5 cpm/pmole = 3×10^6 cpm/ug for a 300 bp fragment labeled at both ends.

Northern hybridization

Unlabeled RNA from 3 ml cultures grown in peptone was extracted as described above and used in the experiment shown in Figure 5.4. Unlabeled RNA prepared as described below for S1 nuclease mapping was treated with pancreatic DNAse according to Branch et al. (1981) and used in the experiment shown in Figure 6.3. Glyoxalation of RNA samples and DNA size markers (McMaster and Carmicheal, 1977), horizontal agarose gel electrophoresis, transfer to nitrocellulose, and hybridization were performed according to Thomas (1980) and Branch et al. (1981), using 3'end labeled DNA probes prepared as above.

S1 nuclease mapping

f1 RNA was prepared from cells grown at 34° or 41.5° in tryptone broth to 1 x 10^8 cfu/ml, infected at moi 100, and harvested at 20 min postinfection. Terminator plasmid RNA was prepared from cells grown at 32° or 37° in tryptone broth + 0.4% galactose + 20 ug/ml ampicillin to 1 x 10^8 cfu/ml. RNA extraction and S1 nuclease mapping were performed as described by Aiba et al. (1981). Restriction fragment probes were 3'end labeled as described above. The probes were subjected to the G and C+T sequencing reactions of Maxam and Gilbert (1980) and electrophoresed as markers beside the S1-resistant DNA samples on 8% acrylamide/7M urea gels in TBE buffer.

Chapter 3
Specific Recombination In Vitro Promoted by the Restriction Endonuclease HgaI

Introduction

The restriction endonuclease HgaI (Takanami, 1973) recognizes the nonsymmetrical five base pair sequence shown in Figure 3.1 and cleaves double-stranded DNA five and ten bases downstream from this site, producing a five base single-stranded end terminating in a 5'-phosphate (Brown and Smith, 1977; Sugisaki, 1978). While the recognition sites and points of cleavage on a given DNA molecule are precisely defined, the base sequence of the single-stranded ends should be unique to each cleavage site. It should be possible to reanneal and ligate only those HgaI fragments that were adjacent in the uncleaved substrate. I have tested this prediction with bacteriophage f1 DNA. f1 RFI is cut by HgaI into six fragments designated A through F in order of decreasing size (Figure 3.2). The cleavage scheme described above predicts that ligation of all six fragments in their original order is the only way to regenerate a circular molecule; such molecules will usually be f1 unit length in size, although production of dimers or higher multimers is possible. I also demonstrate below the highly efficient construction of recombinant f1 phage by this method. In vivo, genetic recombination in f1 occurs at a very low frequency, barely above the reversion rate (10^{-5}) for a single mutation (Lyons and Zinder, 1972). Therefore, f1 crosses in vitro using HgaI should be advantageous in the study of this phage.

Results

Specific ligation in vitro of f1 HgaI fragments

HgaI was purified as described in Chapter 2. Digestion and ligation conditions are also described in detail in Chapter 2. f1 RFI (30 ug) was digested to completion with HgaI and the six fragments produced were separated on a 1.8% agarose gel. After electrophoretic elution from the gel, extraction with phenol, dialysis and precipitation with ethanol, the fragments were mixed in equimolar amounts (0.2 to 0.4 ug f1

Figure 3.1 Cleavage of DNA by HgaI. N represents G, A, C, or T.

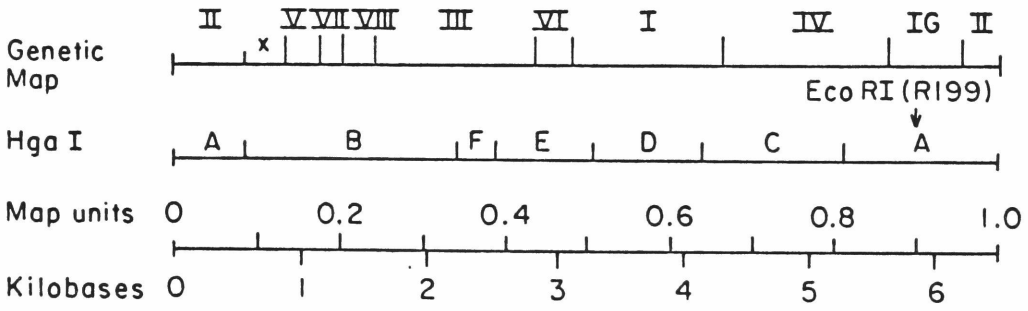


Figure 3.2 Genetic and physical map of the f1 genome. The Roman numerals refer to the genes; IG denotes the intergenic space between genes II and IV which contains the origins of - and + strand DNA synthesis. The HgaI fragments are labeled A through F in order of decreasing size. The sizes of the 6 fragments in base-pairs are 1795, 1638, 1075, 826, 759 and 315 (Beck et al., 1978). The location of the HgaI fragments was determined by gel electrophoretic analysis of fragments produced from HgaI fragments by digestion with HindII, HinfI, HaeIII, and HpaII, and those produced from HaeII fragments by digestion with HgaI, by comparison with the known f1 restriction maps of these 5 enzymes (K. Horiuchi, personal communication). The HgaI maps of f1, fd, and M13 are identical (van Wezenbeck et al., 1980; Beck and Zink, 1981; Hill and Petersen, 1982).

RFI-equivalents each) in pairs and incubated in duplicate tubes with and without T4 DNA ligase. The extent of ligation was determined by electrophoresis of the DNA on a 0.8% agarose gel. Only pairs of fragments originally adjacent on the f1 genome (Figure 3.2) were ligated, and the ligation products were of the expected sizes (Figure 3.3, lanes 2, 4, 6 and 8). No ligation was observed between pairs of fragments which did not share an adjacent HgaI single-stranded end (e.g., HgaI C and E, Figure 3.3, lane 4; HgaI C and F, data not shown), nor in reactions not containing ligase (Figure 3.3, lanes 1, 3, 5 and 7). I conclude that only the HgaI fragments originally adjacent on the f1 genome can be ligated.

Reformation of infectious f1 genomes from HgaI fragments

Based on the above results, one expects ligation of a mixture of the six f1 HgaI fragments to efficiently reform intact f1 DNA molecules. To test this, f1 RFI (3.8 ug) was digested to completion with HgaI and the enzyme was inactivated by heating. One-third of the sample was stored at -20° and the remainder was ligated with T4 DNA ligase. After heating to inactivate the ligase, half the ligated sample was stored at -20° while the rest was redigested with HgaI. Half of each sample was electrophoresed on a 1.8% agarose gel and the other half was used to transfect $CaCl_2$-treated E. coli K38. It can be seen from the gel (Figure 3.4) that ligation of the six f1 HgaI fragments occurred with high efficiency and that the ligated material could be cleaved again with HgaI. The data in Table 3.1 show that digestion with HgaI reduced the number of infectious molecules to background levels, while ligation of the digested DNA restored plaque-forming ability. Inhibition of transfection by high levels of input DNA may account for the non-linearity in number of plaques versus amount of DNA used to transfect cells. Inhibition of transfection by the ligation mixture itself has also been observed (D. Fischhoff, personal communication) and would be more pronounced at the higher DNA concentrations. Based on the control transfection (Table 3.1, plate 1), 2.6 ng of intact f1 RFI would be expected to produce 780 plaques (2.6 ng DNA x 180 plaques/0.6 ng intact f1 RFI). This amount of ligated f1 HgaI fragments produced 23 plaques

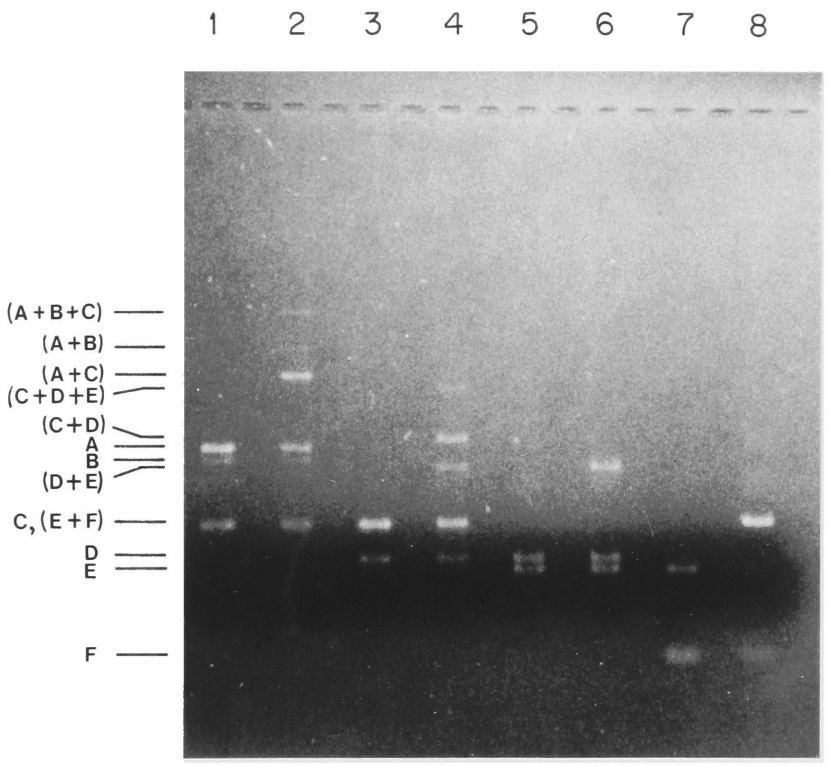


Figure 3.3 Ligation of gel-purified HgaI fragments of f1 RFI. The fragments were prepared as described in the text, and individual fragments were mixed in pairs and incubated, under conditions for ligation described in Chapter 2, in duplicate tubes without (lanes 1,3,5,7) and with (lanes 2,4,6,8) T4 DNA ligase. After the addition of 0.2 vol loading dye (0.1% bromophenol blue, 20% sucrose, 1% SDS, 0.2 M EDTA), the samples were electrophoresed on a horizontal 0.8% agarose gel containing 0.5 ug/ml ethidium bromide at 100 mA/12.5 cm^2 for 5.5 hr and photographed under UV irradiation. The gel-purified fragments HgaI A and D contained small amounts of the closely migrating fragments HgaI B and E, respectively; their presence in the reactions is indicated below in parenthesis. The fragments added to each reaction were as follows: lanes 1 and 2, HgaI A, (B) and C; lanes 3 and 4, HgaI C, D and (E); lanes 5 and 6, HgaI D and E; lanes 7 and 8, HgaI E and F. The positions of the 6 f1 HgaI fragments on the gel are indicated. The ligation products were identified on the basis of size, as determined from mobility on the gel. The order of the HgaI fragments on the f1 genome is shown in Figure 3.2.

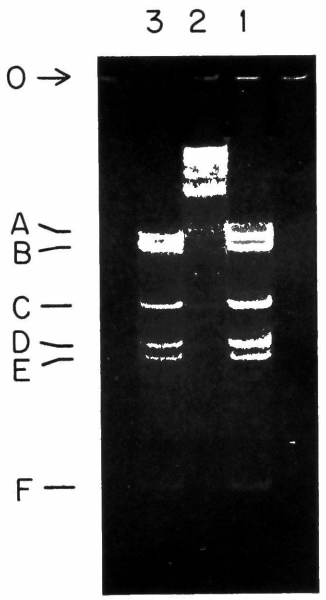


Figure 3.4 Reassembly of f1 RFI digested with HgaI. The conditions for HgaI digestion and ligation by T4 DNA ligase were as described in Chapter 2. The samples were electrophoresed on a vertical 1.8% agarose gel containing 0.5 ug/ml ethidium bromide at 80 V/12 cm for 1 hr 40 min and photographed under UV irradiation. Lane 1, f1 RFI digested with HgaI; lane 2, sample shown in lane 1 after ligation with T4 DNA ligase; lane 3, sample shown in lane 2 after redigestion with HgaI. Each lane contains 0.6 ug f1 RFI-equivalent of f1 DNA. The positions of the f1 HgaI fragments A through F, and the gel origin (O) are indicated.

(Table 3.1, plate 11), 2.9% of the expected value. Therefore about 3% of the HgaI-digested f1 DNA was religated to produce infectious molecules. Reconstruction of infectious f1 genomes by ligation of the six f1 HgaI fragments isolated by electrophoretic separation on an acrylamide gel, and subsequent electrophoretic elution has also been achieved in this laboratory, although at an 80-fold lower efficiency (J. Boeke, unpublished results).

Recombinant genome construction

Construction of recombinant genomes using HgaI should be possible when the relevant mutations lie in different HgaI restriction fragments. Two f1 double amber phage were used to test recombination in vitro with HgaI fragments. The double amber mutants were R30 (gene Vam13 in HgaI B, gene IIam30 in HgaI A) and R79 (gene IVam12 in HgaI C, gene Iam79 in HgaI D). Since each amber mutation maps in a different HgaI fragment, ligation of an equal quantity of HgaI-digested RFI of each parent should yield 1/16 (6.25%) wild type molecules. RFI from each phage was digested with HgaI, mixed in equimolar quantities, and ligated with T4 DNA ligase. Control ligation reactions contained HgaI-digested DNA of one parent only. Samples of the HgaI-digested DNA before and after ligation were electrophoresed on a 0.8% agarose gel to monitor the extent of digestion and subsequent ligation. The initial digestions were essentially complete; after ligation, DNA comigrating with authentic f1 RF was clearly visible (data not shown). The plaque-forming ability of the samples was assayed by transfection of $CaCl_2$-treated E. coli K38 (Su^-) and K37 (Su-1) strains. The data in Table 3.2 show that when a mixture of R30 and R79 HgaI fragments was incubated with ligase, the relative infectivity of the DNA was 0.78% on K37 and 0.059% on K38. Thus 7.6% of the infectious molecules produced were wild type, close to the expected value of 6.25%. Ligated fragments of either parent alone did not produce plaques on K38 (Table 3.2, plates 5 and 6), and the number of plaques due to cotransfection with both parental types was negligible (Table 3.2, plates 3 and 4).

A derivative of f1, R199, containing a unique site for the res-

TABLE 3.1

Infectivity of f1 DNA digested with Hga*I and ligated*

Plate	DNA	ng	$CaCl_2$ cells (ml)	Indicator cells	No. of plaques	% Infectious molecules
1	f1 RFI	0·6	0·3	+	180	100
2	f1 RFI	0·06	0·3	+	27	150
3	f1 RFI	0·6	–	+	1	
4	–		0·3	+	0	
5	*Hga*I-digested f1 RFI	570	0·3	+	1	0·0006
6	*Hga*I-digested f1 RFI	57	0·3	+	6	0·035
7	*Hga*I-digested f1 RFI	3	0·3	+	0	0
8	*Hga*I-digested f1 RFI	3	–	+	0	
9	*Hga*I-digested, ligated f1 RFI	470	0·3	+	374	0·27
10	*Hga*I-digested ligated f1 RFI	47	0·3	+	138	0·98
11	*Hga*I-digested, ligated f1 RFI	2·6	0·3	+	23	2·9
12	*Hga*I-digested, ligated f1 RFI	2·6	–	+	1	
13	*Hga*I-digested, ligated, re-digested f1 RFI	420	0·3	+	2	0·0015
14	*Hga*I-digested, ligated, re-digested f1 RFI	42	0·3	+	0	0
15	*Hga*I-digested, ligated, re-digested f1 RFI	2·3	0·3	+	0	0
16	*Hga*I-digested, ligated, re-digested f1 RFI	2·3	–	+	2	

Infectivity was assayed by transfection of *E. coli* K-38 by the $CaCl_2$ technique (Mandel & Higa, 1970). The number of plaques obtained was normalized against that achieved with 0·6 ng intact f1 RFI (plate 1) as follows:

$$\% \text{ infectious molecules} = \frac{\text{no. of plaques}}{\left(\frac{\text{no. of plaques}}{\text{ng intact f1 RFI}}\right) \times (\text{ng DNA})} \times 100$$

The DNA samples used on plates 5 to 8, 9 to 12 and 13 to 16 correspond to the DNA samples shown in Fig3*A* lanes 1, 2 and 3, respectively.

TABLE 3.2

Infectivity of two f1 double amber mutant DNAs after Hga*I digestion and ligation*

Plate	DNA	ng	$CaCl_2$ cells and indicator cells	No. of plaques	% Infectious molecules
1	(R30 + R79)†	125 each	K-38 (Su^-)	16	0·059
2	(R30 + R79)†	12·5 each	K-38 (Su^-)	0	—
3	(R30) + (R79)‡	125 each	K-38 (Su^-)	0	<0·004
4	(R30) + (R79)‡	25 each	K-38 (Su^-)	1	—
5	(R30)§	250	K-38 (Su^-)	0	<0·004
6	(R79)§	250	K-38 (Su^-)	0	<0·004
7	f1 RFI	0·5	K-38 (Su^-)	54	100
8	(R30 + R79)†	125 each	K-37 (SuI)	359	0·78
9	(R30 + R79)†	12·5 each	K-37 (SuI)	46	1·0
10	(R30)§	250	K-37 (SuI)	185	0·40
11	(R30)§	25	K-37 (SuI)	29	0·63
12	(R79)§	250	K-37 (SuI)	771	1·7
13	(R79)§	25	K-37 (SuI)	90	2·0
14	f1 RFI	0·5	K-37 (SuI)	91	100

Infectivity was assayed as described in the legend to Table 1. The number of plaques obtained was normalized against that achieved with 0·5 ng intact f1 RFI in $CaCl_2$-treated cells of the appropriate bacterial strain, as described in the legend to Table 1. *Hga*I digestion and ligation of the DNA was performed as described in the text.

† *Hga*I-digested RFI of each parental type was mixed and then ligated.

‡ *Hga*I-digested RFI of each parental type was ligated separately and then mixed before transfection.

§ *Hga*I-digested RFI of one parental type was ligated alone.

TABLE 3.3

Construction of amber mutant, EcoRI^S cloning vectors by HgaI-promoted recombination in vitro

	Parent phage	Recombinant phage
R199	EcoRI site in HgaI A	
am8H1	gene VIIIam8H1 in HgaI B	R211 am8H1, EcoRI^S
R24	gene IIam24 in HgaI A gene IVam12 in HgaI C	R214 IVam12, EcoRI^S
R16	gene Vam16 in HgaI B	R215 Vam16, EcoRI^S

triction endonuclease EcoRI was constructed in our laboratory for use as a cloning vector (Boeke et al., 1979). Using the HgaI recombination technique described above, three new f1 variants were created, each containing a specific amber mutation and the EcoRI site of R199. The amber parent of each phage and the relevant HgaI fragments are listed in Table 3.3; see also Figure 3.2). The EcoRI site of R199 is located in HgaI A. After transfection of $CaCl_2$-treated E. coli K37, phage isolates were screened for inability to plate on K38 and for in vivo restriction by K508 (Su-1), which contains the EcoRI-producing plasmid pMB4. EcoRI-sensitive, amber mutant phage were checked for complementation with amber mutants in each of the known f1 genes to ascertain that the amber lesion in the recombinant was that of the amber parent. RFI of each recombinant phage type was subjected to restriction enzyme analysis (data not shown) to confirm the location of the EcoRI site at the position mapped in R199. It is possible, moreover, to deduce the parental origin of HgaI fragments lacking genetic markers if the corresponding fragments of each parent differ in restriction sites for other enzymes. Such differences in the cleavage maps of R199 and am8H1 (a mutant of phage M13, which is closely related to f1) allowed me to determine the source of HgaI fragments A, B, C and E in the recombinant phage R211. HgaI fragments A and C of R211 are derived from R199, while B and E are from am8H1 (data not shown).

Discussion

I have demonstrated the use of HgaI for the efficient construction in vitro of recombinant f1, a bacteriophage for which in vivo recombinants are relatively rare. This technique may be applied to the reassembly of any small DNA molecule possessing HgaI recognition sites, and should be useful for the construction of biologically interesting DNA molecules. As a corollary to this, the HgaI restriction map of a DNA molecule may be ordered based upon the ability of the isolated fragments to be religated.

Chapter 4
Effects of Transposition and Deletion upon f1 Major Coat Protein Gene Expression

Introduction

The proteins encoded by the f1 genome are produced in vastly different amounts in the infected cell. The "cascade mechanism" of f1 gene regulation originally proposed by Okamoto et al. (1969) and further elaborated by Chan et al. (1975), Seeburg and Schaller (1975), Edens et al. (1978), and Konings and Schoenmakers (1978) accounts for the high level of expression of genes VIII and V in vivo as being due to their location proximal to the single rho-independent transcription termination signal. The one-third of the f1 genome covering genes II, V, VII, IX, and VIII contains the five strong G-start promoters and the rho-independent terminator (Figure 4.2). Most of the transcripts from this region have been shown to contain the coding sequences for gene VIII, while gene V is contained on all but the shortest transcripts (Chan et al., 1975; Edens et al., 1978; Cashman and Webster, 1979; LaFarina and Model, 1983). It has been suggested that frequent transcription of genes VIII (major coat protein) and V (single-stranded DNA binding protein) from the upstream G-start promoters may be necessary for their expression at high levels during the phage life cycle. A similar "cascade" has also been suggested for the two-thirds of the f1 genome containing the weaker A-start promoters (Konings and Schoenmakers, 1978), but the distribution of transcripts in this region determined by LaFarina and Model (see Figure 1.1) argues against a "cascade" for the A-start RNAs.

If such "cascades" operated to regulate the levels of f1 gene products, one might expect that relocation of a gene on the circular map would affect its expression, and possibly the expression of other regions of the genome as well. By insertion of a wild type copy of gene VIII into the IG of gene VIII mutant phage to form partial diploids, I demonstrate the functional independence of the gene VIII transcription unit from its position on the f1 genome.

Results

Transposition of gene VIII to the IG

R211 is a gene VIII amber mutant recombinant of M13 and f1 containing a unique EcoRI site in the intergenic space at the junction of f1 HaeIII fragments F and G (nucleotide 5726). It was constructed by recombination in vitro of the genomes of phages R199 and am8H1, using fragments generated by the restriction endonuclease HgaI (Chapter 3). R211 contains the entire am8H1 gene VIII sequence, which is contained within the fragment HgaI B, and the R199 EcoRI site contained within HgaI A (Tables 3.3, 4.1; Figures 3.2, 4.2). I refer to this type of phage as a cp (for coat protein) vector. R211 and am8H1 grow on Su-1 suppressor hosts, which insert serine at amber codons.

The entire gene VIII coding sequence, as well as a strong G-start promoter located 150 bases upstream within gene VII and the rho-independent terminator positioned 42 bases downstream from the last gene VIII coding triplet, are contained within the 829 base pair restriction fragment HpaII C (Figure 4.1). My plan was to insert a wild type copy of this fragment into the EcoRI site of R211 in the hope that the inserted gene would be active enough to confer the ability to grow on a Su^- host, allowing the selection of partial diploid phage carrying two copies of gene VIII. EcoRI sites were created at both ends of this fragment by the biological linker technique of Boeke et al. (1979). The fragment, hereafter called the cp insert, was ligated to EcoRI-cleaved R211 RF and the DNA was used to transfect Su-1 and Su^- hosts. A phage designated R212 was isolated whose genome contained the cp insert located at the former HaeIII F/G border. The orientation of the viral strand of the cp insert (determined by restriction analysis of RF) was the same as in the vector DNA molecule (+ orientation) in 17/17 independent isolates, four of which were obtained in Su-1 hosts in which expression of wild type coat protein from the cp insert should not be required for phage viability. R212 phage grew to roughly equal titers on Su^- and Su-1 hosts, but the plaques formed on both hosts were much smaller than those of wild type f1.

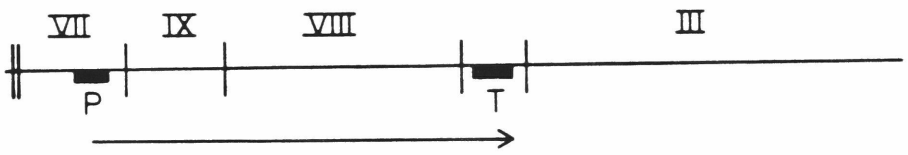


Figure 4.1. Map of the cp insert. This segment of the f1 genome (HpaII C fragment) is 829 bp and contains genes VII, IX, VIII, the carboxy terminal portion of gene V (left side), and the amino terminal portion of gene III. The strong promoter located within gene VII is marked by the heavy bar labeled P. The strong rho-independent transcription termination signal positioned immediately after gene VIII is shown by the heavy bar labeled T. The major transcript from this region in vivo and in vitro is indicated by the arrow (Edens et al., 1978; Rivera et al., 1978; Cashman and Webster, 1979; LaFarina and Model, 1983).

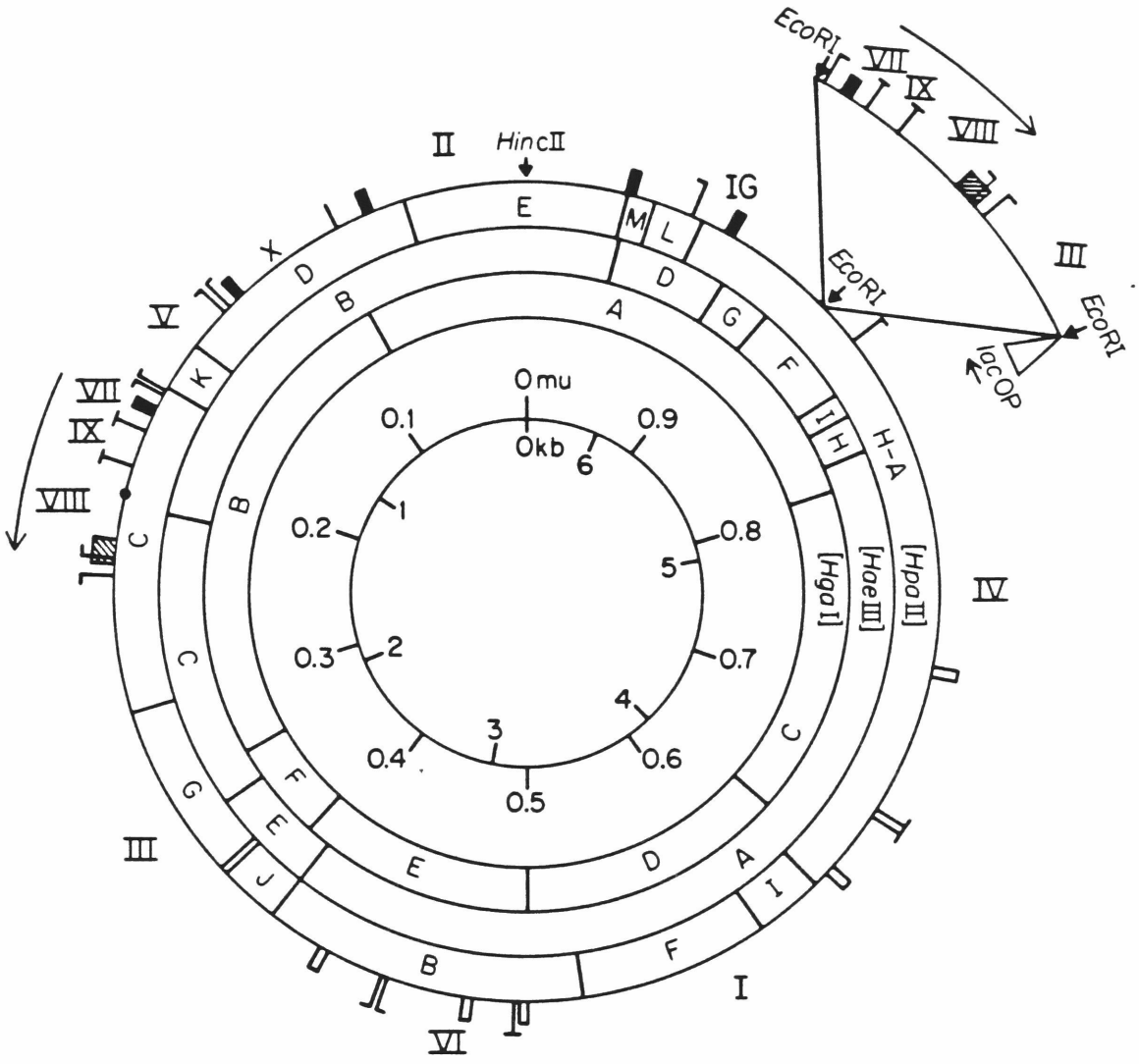


Figure 4.2. Genetic and physical maps of gene VIII partial diploid phage. The outer arc shows the cp insert derived from the DNA fragment HpaII C of wild type f1. The cpb insert also contains the 95 bp E. coli lacUV5 promoter-operator fragment labeled lacOP. The - orientation of the cp(b) insert is shown, but both orientations have been isolated (Table 4.2). Arrows show the direction of transcription on the phage RF. The concentric circles correspond to the genetic map (outer circle), restriction cleavage maps for the enzymes HpaII, HaeIII, and HgaI (middle circles), and scale marked in map units (mu) and kilobases (kb) (inner circle). The single HincII site is taken as the zero position on the map. Genes are indicated by Roman numerals. The solid dot in the gene VIII allele in the normal position marks the location of the coat protein variants. IG denotes the intergenic space between genes IV and II which contains the origins of viral and complementary strand synthesis. The promoter regions and rho-independent transcription termination site are shown on the genetic map: solid boxes = G-start promoters, open boxes = A-start promoters, hatched box = overlapping signals for the rho-independent transcription termination site and the U-start promoter located between genes VIII and III (Schaller et al., 1978).

While it appeared that insertion of the cp insert in the - orientation in R211 was lethal, I hypothesized that this might be due to the self-complementarity of the HpaII C regions on the viral single-stranded DNA, rather than to the failure of expression of gene VIII in the - orientation. Intramolecular double-strand formation might affect DNA replication, since the site of insertion (nucleotide 5726, the HaeIII F/G border) corresponds to the 3'end of the ori-RNA primer for complementary strand synthesis (Geider et al., 1978). I therefore repeated the genome restructuring experiment with a different vector, R229, whose EcoRI site is located at the former HpaII A/H border (nucleotide 5616) on the f1 map (Boeke, 1981). HgaI-promoted recombination in vitro was used to construct new cp vectors with each of the following alleles of the coat gene: am8H1, R240, R258, and R235. The amino acid polymorphisms of these cp variants are listed in Table 4.1. When the cp insert was ligated into the EcoRI site of the amber cp vector R252, selection for phage capable of growth on Su^- hosts yielded partial diploids for gene VIII with both orientations of the cp insert, designated R253 (+ orientation) and R254 (- orientation) (Figure 4.2; Table 4.2).

R254 was further modified by the insertion of the E. coli lacUV5 promoter-operator obtained as a 95 bp EcoRI/PvuII fragment from pGL101 (Guarente et al., 1980), at the gene III end of the cp insert. The EcoRI site at the gene III-lac junction was destroyed by S1 nuclease digestion of the (gene III) single-stranded end before intramolecular blunt-end ligation. The lac promoter in the resulting phage, R269, was oriented with respect to the cp insert such that any transcription originating from lac would proceed in a direction opposite to that of gene VIII transcription (Figure 4.2; Table 4.2). The lacUV5 promoter-operator provides a marker for the cp insert in the absence of wild type gene VIII function. Phage harboring this lac fragment produce blue plaques when plated on media containing the indicator dye 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XG) (Miller, 1972), because they titrate the lac repressor of the host. The cp insert from R269 (cpb insert) was cloned in both orientations in the other cp vectors (Table 4.2).

TABLE 4.1

f1 COAT PROTEIN VARIANTS

Gene VIII Allele	Cp Vector[a]	Gene VIII Codon[c] 2	5	6	12
Wild-type f1	R229; R199	Glu	Asp	Pro	Asp
am8H1	R252; R211	amber	Asp	Ser	Asn
R240	R280	amber	Asp	Ser	Asp
R258	R277	Glu	Asp	Ser	Asn
R235	R270	Glu	His	Pro	Asp
R265[b]	R265	amber	Asp	Phe	Asn
R268[b]	-	amber	Asp	Pro	Asp

[a] EcoRI site at nucleotide 5616 of f1 sequence in all cp vectors except R199 and R211, where it is at nucleotide 5726.

[b] Obtained by UV mutagenesis of gene VIII in vitro. DNA sequence of gene VIII alleles confirmed by restriction analysis (see Figure 4.7) and DNA sequencing.

[c] Codon of mature coat protein.

TABLE 4.2

GENE VIII PARTIAL DIPLOID STRAINS

Partial diploid strain	Parents		Orientation[a]
	Cp Vector	Cp Insert	
R212	R211	f1 HpaII-C	+
R253	R252	f1 HpaII-C	+
R254	R252	f1 HpaII-C	-
R266	R265	R265 HpaII-C-lacOP	+
R267	R252	am8H1 HpaII-C-lacOP	-
R268	R252	R268 HpaII-C-lacOP	+
R269	R252	f1 HpaII-C-lacOP[b]	-
R271	R270	f1 HpaII-C-lacOP	+
R272	R270	f1 HpaII-C-lacOP	-
R273	R270	f1 ThaI/HpaII-C-lacOP[c]	+
R274	R270	f1 ThaI/HpaII-C-lacOP[c]	-
R275	R270	lacOP-f1 ThaI/HpaII-C[d]	+
R276	R270	lacOP-f1 ThaI/HpaII-C[d]	-
R278	R277	f1 HpaII-C-lacOP	+
R279	R252	f1 ThaI/HpaII-C-lacOP[e]	-
R281	R280	f1 HpaII-C-lacOP	+
R282	R280	f1 HpaII-C-lacOP	-

[a] + and - denote orientation of cp insert in IG relative to viral strand of phage

[b] lacOP is a 95 bp EcoRI/PvuII fragment from pGL101 (Guarente et al., 1980) which was ligated to the gene III end of the cp insert (see Figure 4.2 and Results).

[c] ThaI cleavage at nucleotide 1178 of f1 sequence.

[d] lacOP ligated at gene VIII end of cp insert after ThaI cleavage at nucleotide 1178.

[e] ThaI cleavage at nucleotide 1122 of f1 sequence.

Direct proof of the orientation of the cp insert in the viral single-stranded DNA of R253 and R254 was obtained by sequencing one junction of the IG with the cp insert, using f1 restriction fragment HaeIII G as primer in the dideoxy terminator method of Sanger et al. (1977). For R253 100 nucleotides of the IG, the EcoRI site, and 125 nucleotides of the complementary strand of the gene III amino terminus were read. The identical 100 nucleotides of IG and EcoRI site were read for R254, followed by 25 nucleotides of the viral strand of the amino terminus of gene VII (Figure 4.3). These are the sequences predicted from insertion of the cp insert in the + and - orientations respectively. Numerous attempts were made, using several different restriction fragments as primers, to read farther into the cp insert of R254. I have been unable to read more than 25 nucleotides into this region and hypothesize that self-complementarity of the two cp regions in the partial diploid phage DNA may prevent continued DNA synthesis in the sequencing reactions. R254 single-strand preparations exhibit forms on agarose gels which suggest that double-stranded regions are present (data not shown).

The cp insert is an independent transcription unit

Expression of the major coat protein was measured for gene VIII in the normal position on the f1 map and on the cp insert relocated to the IG. For R253, R254, and R212 expression was determined by electrophoresing ^{14}C-phenylalanine labeled whole cell lysates on SDS/urea gels. In Su$^-$ cells only the cp insert is expressed to give full length, functional coat protein, since the am8H1 allele in the normal position should express an amber fragment terminating one amino acid past the signal sequence of pre-coat. In R253 and R254 infected K38 (Su$^-$), coat protein synthesis relative to available transcriptional template (RF) is identical (Table 4.3, lines 1 and 3). Thus, expression from the cp insert is independent of orientation relative to other viral transcription. In K37 (Su-1) cells, both the cp insert and the am8H1 allele are expressed to give full length, mature coat and coat protein synthesis doubles (Table 4.3, lines 2 and 4). Similar results were obtained for R212 (Table 4.3, lines 5 and 6).

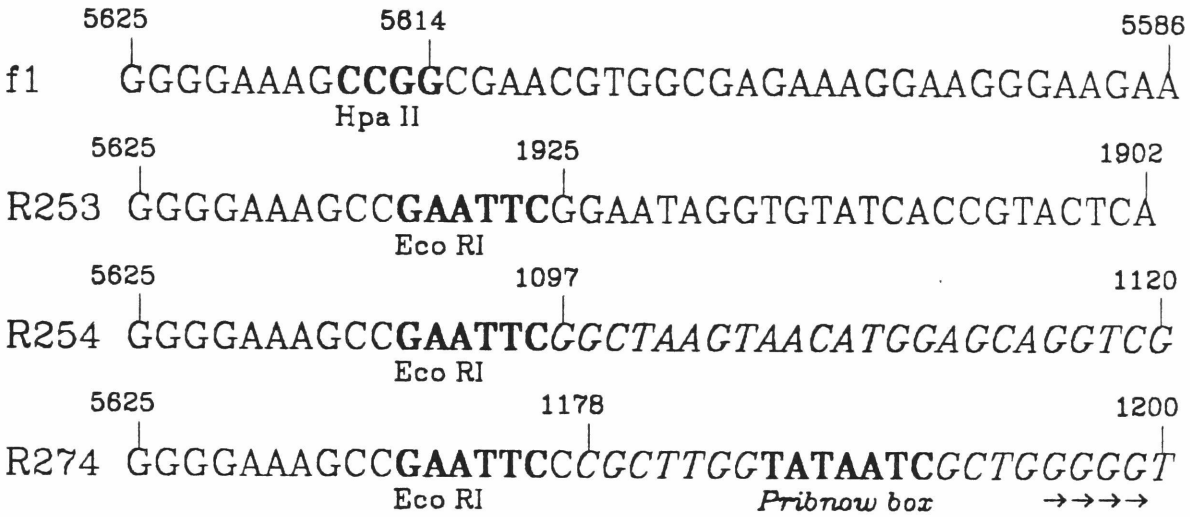


Figure 4.3. DNA sequences at junction of IG and cp inserts in partial diploid phage. Line 1 shows the f1 complementary strand sequence surrounding the HpaII site at position 5614 (Hill and Petersen, 1982). Lines 2, 3, and 4 show the sequences determined for R253, R254, and R274 by the dideoxy terminator method of Sanger et al. (1977) using the restriction fragment HaeIII G as primer. Numbers above the sequences refer to nucleotide position on the f1 map beginning at the unique HincII site. Roman type indicates complementary strand sequences and italic type viral strand sequences; the EcoRI sites at the junctions are in bold face. The Pribnow box (bold face) and initiation nucleotides (->) of the gene VIII promoter are present on the R274 cpb insert; the upstream -35 promoter sequence is deleted.

R271 and R272 are partial diploids containing the R235 and wild type f1 alleles (Tables 4.1, 4.2). R235 coat protein differs from wild type f1 only at amino acid five (histidine vs. aspartic acid), migrates faster in SDS/urea gels, and can be completely resolved from f1 coat (Boeke et al., 1980). Figure 4.4 shows the autoradiogram of a gel on which lysates of ^{14}C-phenylalanine labeled R271 (lane 7) and R272 (lane 8) infected cells were electrophoresed. The R235 and wild type f1 coat proteins synthesized from the normally positioned and relocated copies of gene VIII, respectively, were quantitated by microdensitometer scanning. Similar expression of the two gene VIII copies was obtained irrespective of location or orientation (Table 4.4, lines 1-4).

In order to exclude the possibility that transcription originating in or reading through the IG was responsible for expression of the cp insert, I deleted part of the promoter and measured coat synthesis from the truncated cp insert. The gene VIII promoter sequence closely resembles the consensus prokaryotic promoter sequence in both the conserved -35 and -10 or Pribnow box regions (Figure 5.6) (reviewed by Rosenberg and Court, 1979). A ThaI site separates these regions at nucleotide 1178 of the f1 sequence. The cpb insert was cut with ThaI to remove 81 bp from its 5'end and a new EcoRI site was created at that end by ligation of a synthetic linker. The fragment was recloned in the cp vector R270 and phage making blue plaques on XG plates were isolated. The genomic structure of these phage, R273 and R274, was confirmed by restriction analyses of RF and dideoxy sequencing of viral single-stranded DNA (Figure 4.3) and found to be as predicted: identical to R271 or R272 except for the absence of 81 bp from the promoter end of the recloned fragment. Coat synthesis by R273 and R274 was determined by ^{14}C-phenylalanine labeling of infected cells (Figure 4.4, lanes 5, 6, 9, and 10; Table 4.4, lines 5-8). Coat protein with the R235 type mobility is present. No coat protein with the wild type f1 mobility is detectable (compare R271 and R272, lanes 3, 4, 7 and 8). Deletion of the -35 region of the gene VIII promoter appears to entirely destroy expression of coat protein from the cp insert in vivo.

Partial restoration of coat synthesis can be achieved by replacing

TABLE 4.3

DNA AND COAT PROTEIN SYNTHESIS IN PARTIAL DIPLOID STRAINS

Partial diploid phage strain[a]	Host cell[b]	RF[c]	Single strands[c]	Coat protein synthesis relative to wild-type f1 control = 1.00[d]
R253 +	K-38	0.78	0.88	0.34 (0.43)
	K-37	0.89	0.94	0.84 (0.94)
R254 -	K-38	0.55	0.19	0.23 (0.42)
	K-37	0.70	0.37	0.54 (0.77)
R212 +	K-38	0.11	0.09	0.10 (0.91)
	K-37	0.06	0.10	0.16 (2.67)

[a] See Table 4.2.

[b] K-38 is Su^-; K-37 is Su-1, serine-inserting amber suppressor.

[c] Intracellular levels at 30 min postinfection expressed relative to wild-type f1 control = 1.00. Measured by sucrose gradient analysis of ^{3}H-thymidine-labeled infected cultures as described in Chapter 2.

[d] Average of 3 to 6 determinations at 30 min postinfection as described in Chapter 2. Numbers in parentheses = coat protein/RF.

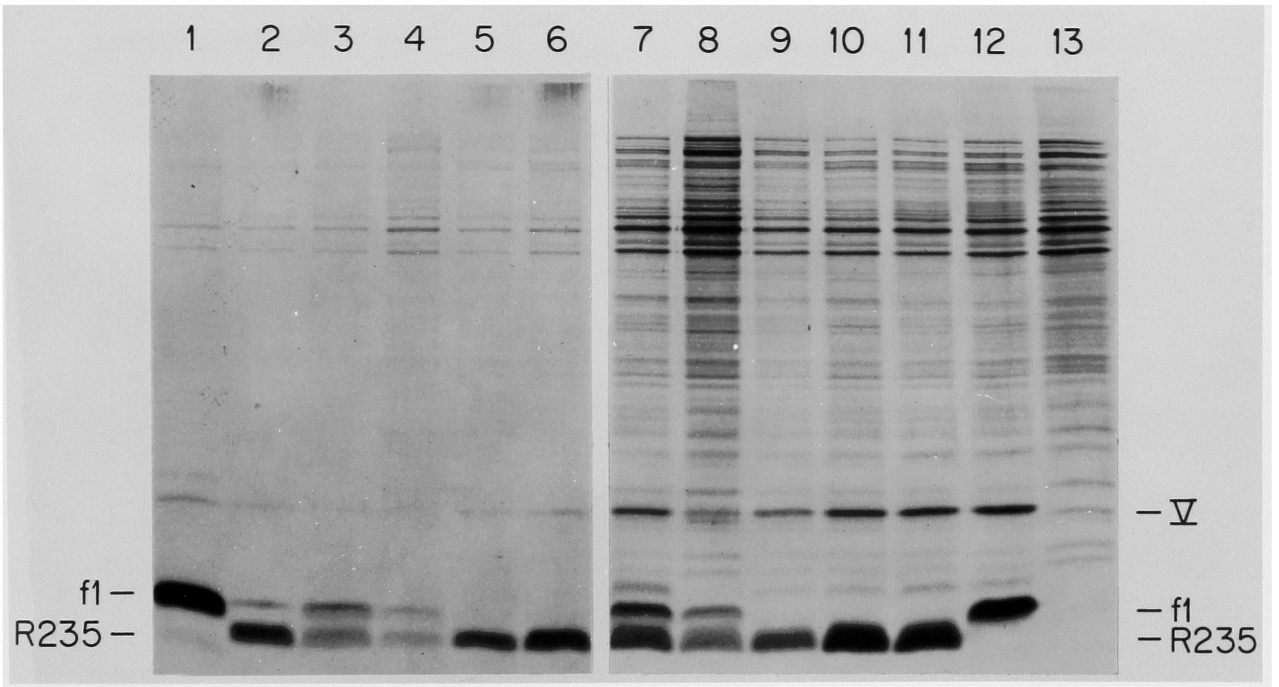


Figure 4.4. Coat protein synthesis in partial diploid phage containing R235 and wild type f1 coat protein gene copies. K38 cells were infected and labeled with ^{14}C-phenylalanine as described in Chapter 2. Electrophoresis was on an SDS/urea gel containing 23% acrylamide/0.088% bisacrylamide. Lanes 1 through 6 contain disrupted extracellular virions (supernatant fraction) from the labeled cultures; exposure time was 14 days. Lanes 7 through 13 contain cellular extracts (pellet fraction); exposure time was 6 days. The positions of wild type f1 and R235-type coat proteins are indicated. Infected cultures were as follows: wild type f1, lanes 1 and 12; R270, lanes 2 and 11; R271, lanes 3 and 7; R272, lanes 4 and 8; R273, lanes 5 and 9; R274, lanes 6 and 10; uninfected K38, lane 13. The position of the phage specified gene V protein is indicated on the right. This protein, a single-stranded DNA binding protein, is localized intracellularly; thus its presence in lanes 1 through 6 indicates some cellular contamination of the virion fractions. The presence of some wild type f1 coat protein in lane 2 is due to leakage from lane 1; in the corresponding intracellular samples (lanes 11 and 12) no leakage occurred.

TABLE 4.4

COAT PROTEIN SYNTHESIS FROM TRANSPOSED AND DELETED GENE COPIES

Partial diploid phage strain[a]	Coat gene Position	Coat gene Allele	Fraction of coat protein represented by each allele[b] Intracellularly	Fraction of coat protein represented by each allele[b] In virions
R271 +	IG	f1	0.37	0.44
	Normal	R235	0.63	0.56
R272 -	IG	f1	0.35	0.42
	Normal	R235	0.65	0.58
R273 +	IG	f1	0	0
	Normal	R235	1.0	1.0
R274 -	IG	f1	0	0
	Normal	R235	1.0	1.0
R275 +	IG	f1	0.1	0.1
	Normal	R235	0.9	0.9
R276 -	IG	f1	0.1	0.1
	Normal	R235	0.9	0.9

[a] See Table 4.2

[b] Average of 1 to 3 determinations at 30 min postinfection as described in Chapter 2. Host cells were K-38.

the deleted sequence with the lacUV5 promoter (Table 4.4, lines 9-12) or with DNA homologous to the consensus -35 sequence (Chapter 5). Deletion of only 25 bp rather than 81 bp from the 5'end of the cpb insert, up to nucleotide 1122 of the f1 sequence, does not destroy promoter function in phage R279, otherwise analogous to R269 and R274 (data not shown).

I conclude from the above experiments that gene VIII with its most proximal promoter and terminator contained on the cpb insert behaves as an independent transcription unit of the f1 genome.

The position and orientation of the cp insert affects f1 phage production

R253, R254, and R212 have markedly reduced growth rates relative to wild type f1 (Figure 4.5). R212 is the most severely affected; phage production does not commence until 50 min postinfection, compared to 30 min for wild type f1, and less than 10% the wild type number of phage are released over a three hour period. R254 infected cells release phage at 25% the rate of wild type f1 infected cells. K38 (Su^-) infected by R253 initially release phage at 50% the rate of a wild type infection, but phage production levels off at later times. R253 growth is markedly improved in the K37 (Su-1) host, increasing to 75% the wild type level and remaining high for two hours. In contrast, growth of R254 and R212 is not improved in the Su-1 host.

R253, R254, and R212 produce mature coat from the cp insert only in K38, while in K37 both the cp insert and am8H1 gene VIII copies are expressed to give mature coat. Thus it seems that increasing the gene dosage of coat protein increases the number of phage released by R253 infected cells, while no such effect is observed for R254 or R212 infected cells. Some other factor must be limiting the rate of production of the latter phages despite the fact that the only difference between the three phage strains is the location and orientation of the cp insert in the IG.

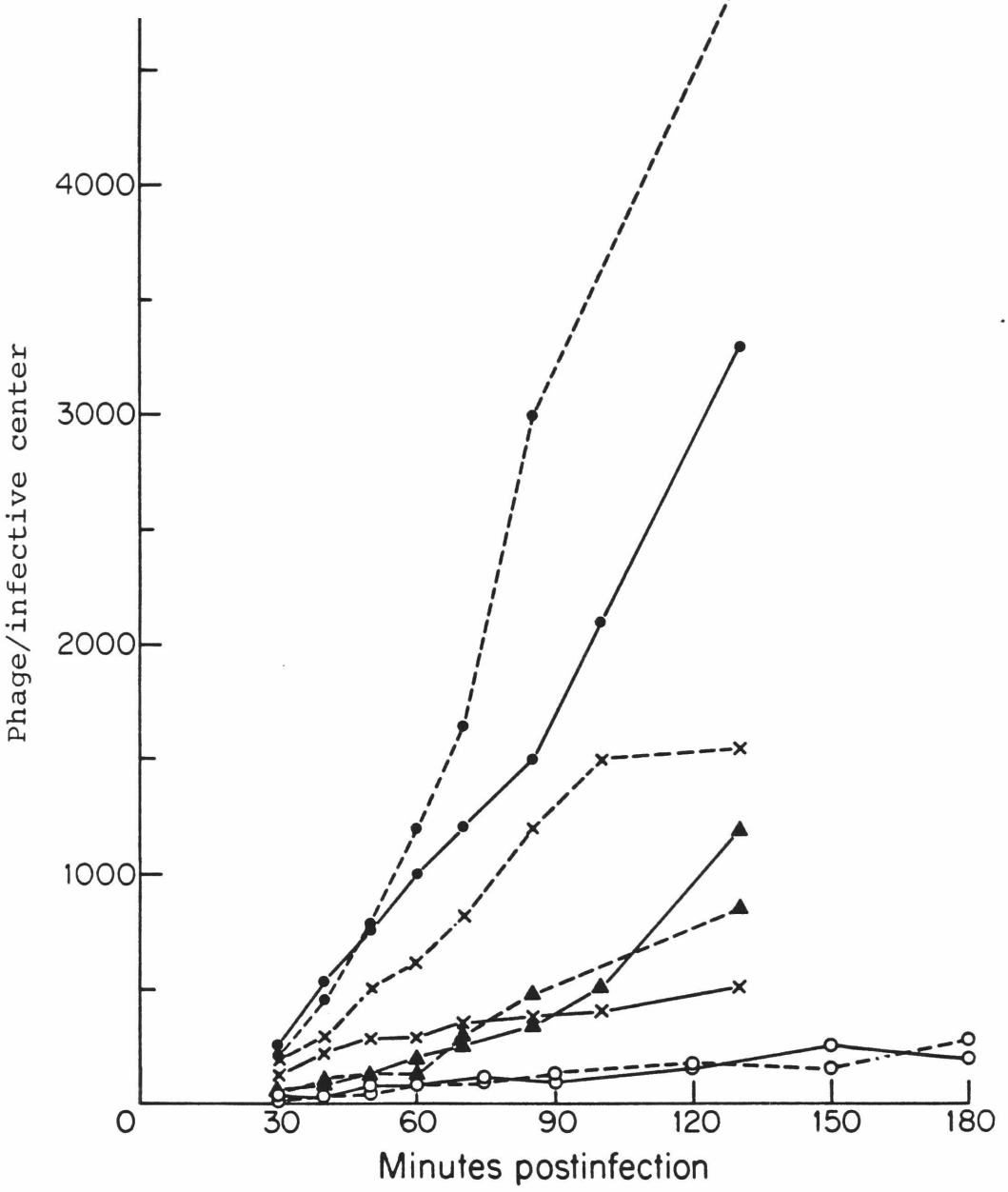


Figure 4.5. Kinetics of partial diploid and wild type f1 phage production. Phage production in K38 and K37 hosts was measured by infecting cultures grown at 37° in DO salts (Vogel and Bonner, 1956), 0.5% glucose, 5 ug/ml thiamine, and 1-2 mM amino acids (minus phenylalanine) to a density of about 6 x 10^8 cfu/ml at moi 16. Phage were adsorbed for 8 min, a portion of each culture was diluted into f1 antiserum to inactivate unadsorbed phage, and infective centers were plated on K38. The remainder of each culture was diluted to prevent further phage infection and phage production was assayed at various times by sampling the cultures into tryptone broth plus chloroform and plating on K38. Infective centers were determined at the time of infection only and are not corrected for subsequent increase in cell titer. The doubling time of uninfected cells in DO medium is 40 min and increases about 2-fold upon f1 infection. Input phage were 20-50 per infective center at the time of infection and are not subtracted from the values plotted.—K38 (Su^-) host; ---- K37 (Su^+) host; • wild type f1; x R253; ▲ R254; o R212. Values are the average of 1-3 determinations.

The position and orientation of the cp insert affects RF and SS DNA synthesis

RF and single-strand (SS) DNA levels intracellularly at 30 min postinfection were measured by sucrose gradient analysis for R253, R254, and R212 in K38 and K37 hosts (Table 4.3). R212 RF and SS synthesis is reduced to 10% of the wild type f1 level. The R254 RF level is 60% of wild type, while the SS DNA level is 25%. RF and SS levels are both 90% of wild type in R253 infections. I conclude that cloning the cp insert in the IG can have marked effects on DNA replication, dependent upon the site of insertion and the orientation of the fragment with respect to the viral genome. Note that the - orientation counterpart of R212 could not be isolated.

f1 virions can be assembled using coat protein monomers of different charge and amino acid composition

The variant coat alleles listed in Table 4.1 differ from wild type near the amino terminus. Reactivity to antiserum is affected by these amino acid polymorphisms (Pratt et al., 1969; Wickner, 1976). I wanted to know whether domains of the coat protein involved in assembly were also affected. Therefore, I investigated whether the variants coassembled with wild type molecules in partial diploid virions, or whether two separate populations of progeny virions could be distinguished. I analyzed the coat protein composition of mature virions in several ways as described below.

When intact f1 virions are electrophoresed on a 2% agarose gel in 0.37 M Tris-glycine buffer (pH 9.5), their mobility is determined by the length of the virion, the net charge of the major coat protein molecules, and their secondary/tertiary structure (Beaudoin, 1970; Boeke et al., 1980). The length of an f1 virion is determined by the size of the single-stranded DNA molecule encapsidated (Enea and Zinder, 1975; Enea et al., 1977). Therefore, R253 phage grown in the Su^- host migrate more slowly than f1 wild type phage, due to the 839 base cp insert (Figure 4.6, lanes 1 and 2). f1, am8H1, R240, R277, and R270, which possess DNA molecules of almost identical size, have different mobilities in

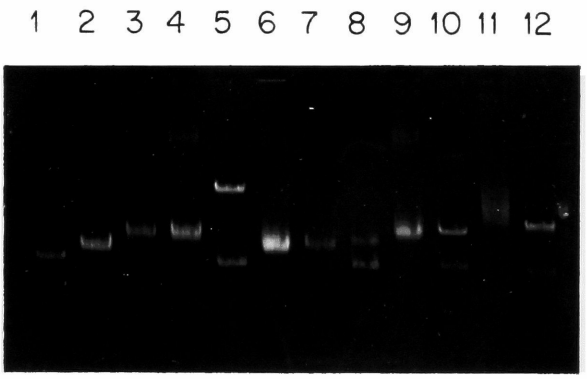


Figure 4.6. Agarose gel electrophoresis of intact virions. Host strains are indicated in parentheses. Lane 1) f1 (K38) 2) R253 (K38) 3) R253 (K37) 4) R254 (K37) 5) f1 (K38) plus am8H1 (K37) 6) R282 (K38) 7) R282 (K37) 8) f1 (K38) plus R240 (K37) 9) R278 (K38) 10) f1 (K38) plus R277 (K38) 11) R271 (K38) 12) f1 (K38) plus R270 (K38). Electrophoresis was on a 2% agarose gel in 0.37 M Tris-glycine pH 9.5 (Beaudoin, 1970). Approximately 1-2 x 10^{10} virions were applied in each lane. The positions of the virions were visualized by staining the viral DNA with ethidium bromide (Nelson et al., 1981).

this gel system (Figure 4.6, lanes 5, 8, 10, and 12), due to different net charges of the major coat protein (Table 4.1). When the variant coat genes are expressed in addition to the wild type f1 coat gene in the partial diploids, the mobility of the virions is shifted from that of phage expressing wild type f1 coat only towards that of phage with the variant allele only. The diploid coat combinations am8H1/f1, R240/f1, and R258/f1 give single sharp phage bands at mobilities between the haploid parents (lanes 3, 4, 7, and 9), in contrast to samples containing a mixture of virions of each of the two parental types, which resolve into the two parental bands on the gel (lanes 5, 8, and 10). The diploid coat combination R235/f1 produces a broad band which is not seen when R235 and wild type f1 virions are mixed (lanes 11 and 12). The altered mobilities seen for each partial diploid strain suggest that each virion has a capsid composed of a mixture of the two types of gene VIII protein monomers coded for by its DNA.

Direct amino acid analysis of capsid protein from virions was used to quantitate the ratio of am8H1 to wild type f1 coat monomers in R253, R254, and R212. Mature gene VIII protein of wild type f1 contains a single proline residue at position six, which is replaced by serine in am8H1 coat protein (Table 4.1). The amino acid compositions of partial diploid virions grown on serine-inserting hosts were determined and compared to those of wild type f1 and R252 (am8H1 allele). The ratios of serine, glutamic acid/glutamine, and proline relative to aspartic acid/asparagine, which are equimolar in f1 and am8H1 coat, were determined for each phage strain and compared to those predicted for wild type f1 coat, am8H1 coat, and three possible ratios of the two coat monomers (Table 4.5). The data suggest that R253, R254, and R212 virions all contain am8H1:wild type f1 coat monomers in the ratio 1:2 or 1:3.

R235 coat protein can be completely resolved from wild type f1 coat protein on acrylamide gels (see above). ^{14}C-phenylalanine labeled R271 and R272 virions were disrupted and electrophoresed. The R235 type and wild type f1 coat proteins in each virus population and in the infected cells (Figure 4.4, lanes 3, 4, 7, and 8) were quantitated by mi-

TABLE 4.5

EXPERIMENTAL AND PREDICTED MOLE RATIOS OF

AMINO ACIDS IN GENE VIII PROTEINS

	Experimental[1]					Predicted[2]				
								am8H1:f1 protein molecules		
	f1	R252 (am8H1)	R253	R254	R212	f1	am8H1	1:1	1:2	1:3
Ser/Asx	1.24	2.15	1.35	1.30	1.37	1.33	2.0	1.67	1.56	1.50
Glx/Asx	1.07	0.70	0.87	0.87	0.93	1.0	0.67	0.83	0.89	0.92
Pro/Asx	0.33	0.09	0.24	0.25	0.23	0.33	0	0.17	0.22	0.25

[1] Determined from amino acid analysis of virion capsid protein, >99% of which is gene VIII protein.

[2] Based on the following moles of amino acids per mole of mature gene VIII protein (Nakashima and Koningsberg, 1974; Boeke and Model, 1979):

	f1	am8H1
Ser	4	6
Glx	3	2
Pro	1	0
Asx	3	3

crodensitometer scanning. The relative amount of each in both R271 and R272 virions was found to be approximately 57% R235 type, 43% f1 wild type. This reflects the intracellular ratio of the gene VIII proteins synthesized (Table 4.4, lines 1-4).

New alleles of gene VIII

Two new viable variants of the am8H1 allele have been created by UV mutagenesis of the cpb insert in vitro, followed by religation to unmutagenized R252 vector RF and transfection of unirradiated Su-1, recA$^+$ hosts (protocol described in Chapter 2). Phage making blue plaques on XG plates were isolated and screened for the amber phenotype (inability to plate on Su$^+$ hosts). Three isolates with full-length cpb inserts were characterized by restriction analysis, and Maxam-Gilbert DNA sequencing of the cpb inserts. The amino acid and DNA sequence polymorphisms at positions 5, 6, and 12 are shown in Table 4.1 and Figure 4.7, and are distinguishable by the presence or absence of MboI (Sau3A) and HinfI sites in the DNA (Boeke et al., 1980). The partial diploid R267 was found to contain the am8H1 gene VIII sequence at both the normal position and on the cpb insert. R268 contained the am8H1 allele at the normal position and a new, recombinant allele on the cpb insert. The R268 allele was amber at codon 2, but wild type f1 at codons 5, 6, and 12. R266 contained new, identical gene VIII alleles at the normal position and on the cpb insert. The haploid R265 was derived from R266 by excision of the cpb insert with EcoRI, followed by religation of the vector molecule in vitro, and transformation of Su-1 cells with the ligated DNA. The R265 coat gene was sequenced by the dideoxy terminator method, and found to code for phenylalanine at position 6, with codons 2, 5, and 12 the same as in am8H1. Thus, allowable residues at position 6 include proline, serine, and phenylalanine, amino acids with very different properties.

Discussion

The new gene VIII alleles may have been created by gene conversion in the partial diploid phage, which were isolated in recA$^+$ hosts. For R267 and R268, recombination between the wild type f1 gene copy on the

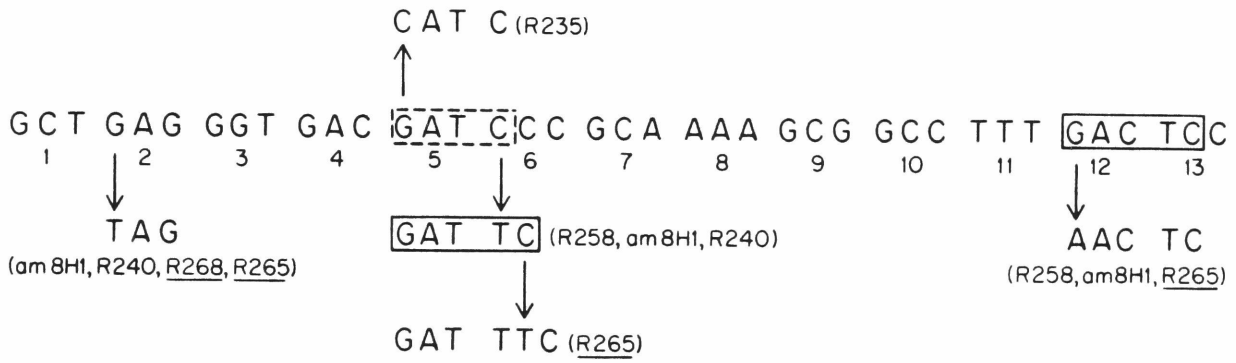


Figure 4.7. DNA sequence changes in new alleles of gene VIII. The wild type f1 sequence coding for amino acids 1 through 13 of coat protein is shown, with coat protein variant mutations indicated above and below. Solid boxes enclose HinfI sites, broken box encloses MboI (Sau3A) site (Boeke et al., 1980). The new variant alleles R265 and R268 were isolated as described in the text and characterized by restriction analysis and DNA sequencing.

cpb insert and an am8H1 gene copy on the same or a different RF molecule could explain the replacement of the f1 sequence on the cpb insert with the am8H1 or new, recombinant R268 allele. For R266, an intramolecular event seems most likely. Inaccurate repair of a UV-induced lesion on the cpb insert, using the am8H1 gene copy as a template, could have resulted in the conversion of both copies to the new sequence, which differs from the am8H1 sequence by a single C->T transition following a run of two T's (Figure 4.7). Analysis of UV-induced mutations in the lacI gene (Miller, 1982) and in the lac promoter (LeClerc and Istock, 1982) have shown the most common event to be a GC -> AT transition in which the mutated base pair is part of a potential pyrimidine-pyrimidine dimer. The R266 mutation is this type of event.

My experiments show that the gene VIII promoter, coding sequence, and terminator contained on the f1 HpaII C fragment (cp insert) comprise an independent transcription unit which expresses coat protein at a high level regardless of its position or orientation relative to other viral transcription, but dependent upon its own intact promoter. The results demonstrate that transcription of f1 RF can proceed in both directions relative to the viral strand, when the appropriate regulatory signals are present. I have found that the -35 sequence upstream of the Pribnow box is crucial for promoter function in vivo, a result which contrasts with previous in vitro experiments (Okamoto et al., 1977). Transcription of the coat gene from the four other upstream promoters (Figure 4.2) is not required for phage growth. Thus, the "cascade model" is not needed to explain the high level of coat synthesis, which must approximate 10^6 molecules/cell/generation (2.7×10^3 molecules/phage x 10^3 phage/cell/generation in a wild type infection; see Figure 4.5).

I therefore suggest that the large amount of coat protein produced during the f1 phage infection cycle is not a direct consequence of the map location of gene VIII, but is probably regulated by other factors, such as a high frequency of transcription from the most proximal promoter, stability of the gene VIII mRNA, and the frequency of translation of the message. A 370-nucleotide RNA coding for gene VIII has been found to comprise as much as 2% of the RNA transcribed in f1 infected

cells (Rivera et al., 1978; Cashman and Webster, 1979), and a component of gene VIII mRNA has been shown to possess a long functional half-life (La Farina and Model, 1978; Rivera et al., 1978). It has also been demonstrated that coat protein is not efficiently translated in vitro from mRNAs isolated in vivo which code for both genes V and VIII, when compared to mRNA which codes only for gene VIII (Cashman and Webster, 1979).

While gene VIII is expressed at equal levels from its transcription unit in both orientations when the site of insertion is nucleotide 5616 of the f1 sequence, insertion in the - orientation at nucleotide 5726 is lethal. This may be due to deleterious effects upon DNA replication, since this position corresponds to the 3'end of the ori-RNA primer for complementary strand synthesis (Geider et al., 1978). DNA replication is also adversely affected by the - orientation insert at position 5616. These effects may be caused by the self-complementarity of the HpaII C regions (13% of the wild type genome length) in the viral single-stranded DNA. However, these DNA molecules are efficiently packaged into infectious virions.

The low rates of DNA synthesis in R254 and R212 infections, which appear to be caused by the cp insert in the IG, correlate with the kinetics of phage production. Increasing the supply of coat protein by suppression of the am8H1 allele in Su-1 hosts does not improve phage production; in these infections DNA synthesis seems to be the limiting factor. In contrast, DNA levels are near wild type in R253 infections, but coat protein synthesis is limiting in Su^- hosts. Increasing the coat protein supply in Su-1 hosts allows a higher rate of phage production. Thus the rate of f1 phage production is determined by two factors at least, and these appear to be in an optimal ratio in wild type infections.

Full-length coat protein synthesis in R253 and R254 infected Su^- hosts is only one-half of the f1 value based on the level of transcriptional template (RF) (Table 4.3). Translation of mRNA from the am8H1 gene copy is also occurring but terminates at codon 2 (codon 25 of pre-

coat). Possible explanations are: 1) The cellular translational machinery might be limiting, so that the total amount of gene VIII mRNA available cannot be utilized for coat protein synthesis. 2) The transcriptional capacity of the host cell might be limiting. 3) The large amount of amber coat fragment synthesized is detrimental to the infected cells. In R212 infected Su^- hosts full-length coat synthesis is proportional to RF. These levels are only one-tenth of wild type, and so the effects considered above might not occur.

I have shown that virions can tolerate the combination of wild type f1 coat monomers with any of several variant coat alleles, and that the average virion coat composition reflects the pool of coat monomers synthesized intracellularly. Pratt et al. (1969) have shown phenotypic mixing between f1 and M13 coat proteins. The amino acid polymorphisms seen in the coat protein variants thus far isolated all occur near the amino terminus of the mature coat protein. This portion of the molecule interacts with the solvent at the surface of the phage particle (Webster and Cashman, 1978). Presumably specific protein-protein or protein-DNA interactions are not as important in this region of the protein as in the hydrophobic central portion or basic carboxy terminus.

Chapter 5
Structural Instability of f1 Gene VIII mRNA Containing *lac* mRNA Sequence at the 5'end

Introduction

Two major factors contributing to the enormous production of the f1 major coat protein in vivo appear to be the strength of the most proximal gene VIII promoter (Seeberg and Schaller, 1975; Okamoto et al., 1975; Edens et al., 1976; Seeburg et al., 1977) and the accumulation of large amounts of the 369 nucleotide gene VIII mRNA, which has a long (~10 min) functional and structural half life (LaFarina and Model, 1978; Rivera et al., 1978). In contrast, the 2000-nucleotide mRNA coding for another f1 protein, gene II protein, has a short (<1 min) half life and is detected only at low levels, although it is transcribed from a very efficient promoter (Seeburg and Schaller, 1975; Okamoto et al., 1975; Edens et al., 1976; Seeburg et al., 1977; Cashman et al., 1980; LaFarina and Model, 1983). The 2000- and 369-nucleotide mRNAs are 3'coterminal: both contain gene VIII coding sequences at their 3'termini, but they differ at their 5'ends, where gene II is encoded on the 2000-nucleotide mRNA. Typical mRNAs such as the E. coli *lac*, *gal*, and *trp* operon mRNAs are unstable (1-2 min half lives), and are thought to decay in the 5'->3' direction (reviewed by Miller and Reznikoff, 1978; Lim and Kennell, 1979).

I therefore thought it possible that the 5'end sequences of an mRNA might influence its stability. For the study described here, gene VIII was placed under the control of the *lac*UV5 promoter such that a hybrid mRNA containing the 5' 36 nucleotides of *lac* mRNA joined to the entire sequence of gene VIII mRNA was transcribed. This mRNA, which can be translated to give coat protein, is unstable and decays with *lac* kinetics in vivo.

Results

Construction of a hybrid lac-gene VIII transcription unit

A hybrid *lac*-gene VIII transcription unit was constructed in vitro

by replacement of f1 nucleotides 1095-1177 on the wild type cp insert (Chapter 4) with the lacUV5 operator-promoter, obtained on a 95 bp EcoRI/PvuII fragment from pGL101 (Guarente et al., 1980; Boeke et al., 1982). This hybrid fragment, hereafter called the lacVIII tu (Figure 5.1), was cloned in pBR322 and in the cp vector R270 (Chapter 4) at their respective EcoRI sites. The plasmid clones pJB1 and pJB2 contained the lacVIII tu in opposite orientations, and both expressed f1 gene VIII from the lac promoter, i.e., coat protein was made in vivo and its production was stimulated by the addition of the lac inducer IPTG to the medium (Boeke, 1982). However, coat protein synthesis could not be completely shut off, even in the lac repressor overproducing host K561 (i^Q). The partial diploid phages R275 and R276 contained the lacVIII tu in opposite orientations in the f1 IG (Table 4.2, lines 12, 13) and both produced wild type f1 coat protein at approximately 10% the level obtained from the coat gene in the normal position (Table 4.4, lines 9-12). It was shown in Chapter 4 that deletion of the -35 region of the gene VIII promoter eliminated coat protein expression entirely from the cp insert in the IG of phages R273 and R274 (see Table 4.2, lines 10, 11; Table 4.4, lines 5-8; Figures 4.3, 4.4). R273 and R274 differ only in the orientation of the truncated cp insert in the IG. These results, taken together with the results cited above, indicate that coat protein expression in the phages and plasmids cannot be attributed to transcription from irrelevant upstream promoters present on the vector molecules, but must be caused by transcription from an active promoter on the lacVIII tu itself.

Identification of mRNAs from the lacVIII tu

A hybrid lacVIII mRNA of 423 nucleotides would be expected from transcription initiating at the lac promoter of the lacVIII tu. This mRNA should contain the first 36 nucleotides of lac mRNA, 18 nucleotides from f1 gene VII, and the complete 369 nucleotide gene VIII transcript ending at the f1 rho-independent terminator. I show below that such a lacVIII mRNA is produced both in vivo and in vitro from the lacVIII tu.

Plasmid-containing, or phage-infected K38 or K561 (i^Q) cells were

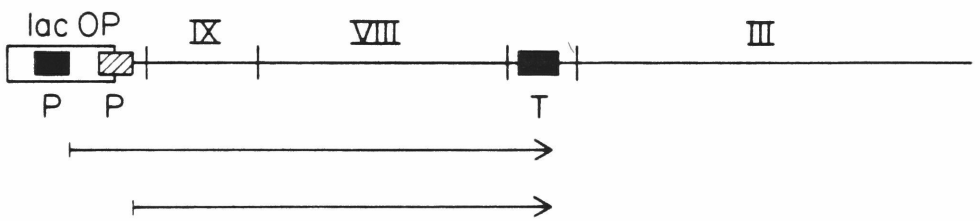


Figure 5.1. Map of the lacVIII tu. This transcription unit consists of the 95 bp EcoRI/PvuII fragment from pGL101 containing the lacUV5 operator-promoter (open segment) fused to f1 nucleotide 1178, just upstream of the Pribnow box of the gene VIII promoter on the cp insert (thin line segment; see Figures 4.1 and 5.6). f1 genes IX, VIII, and the amino terminus of III are contained on the fragment. The f1 rho-independent terminator is located between genes VIII and III (solid box T), overlapping with the weak gene III promoter. The lacUV5 promoter (solid box P) and fortuitous lacVIII-promoter created by the fusion (hatched box P; see text and Figure 5.6) are shown along with the expected 420- and 370-nucleotide RNAs, which were identified as described in this Chapter.

grown in peptone, labeled with ^{32}P-phosphate, and the RNA extracted and electrophoresed on 4% polyacrylamide gels in TBE buffer. K38 harboring pJB1 or pJB2 contain two new RNA species whose sizes as estimated from RNA standards are 420 and 370 nucleotides (Figure 5.2, lanes a-g). The 420-nucleotide RNA is barely detectable in uninduced K561 (i^Q) harboring these plasmids (lanes h and j), but is restored by the addition of IPTG to the growth medium (lanes i and k).

f1-infected cells contain an RNA which migrates slightly faster than the 420-nucleotide lac-inducible RNA of pJB1 and pJB2 (Figure 5.3, lanes b-e). Smits et al. (1980) and Cashman et al. (1980) have characterized an f1 RNA of this size which appears to be a processed species containing gene VIII coding sequences and ending at the rho-independent terminator. R276-infected cells contain this processed f1 gene VIII RNA, but they also contain the 420-nucleotide lac-inducible RNA found in pJB1 and pJB2 (Figure 5.3, lanes f-i). The presence of the processed f1 gene VIII RNA makes it more difficult to see the lac-inducible RNA in R276, compared with pJB1 and pJB2, which lack the processed f1 gene VIII RNA species. However, the 420-nucleotide lac-inducible RNAs of pJB1, pJB2, and R276 comigrate on the gel (Figure 5.2, compare lanes h-k with lane l). The 420-nucleotide lac-inducible RNA is absent in wild type f1-infected cells (Figure 5.3, compare lanes b-e with f-i).

Northern hybridization analysis of unlabeled RNA from cells harboring R276 or pJB2 showed a ~420-nucleotide species that reacted with a ^{32}P-labeled lacOP probe (Figure 5.4, lanes i,j). This RNA was not detected in cells harboring f1 or pBR322 (lanes h,k). The longest RNAs from cells harboring pJB2 which reacted with a gene VIII probe were about 450 nucleotides (lane c). In contrast, f1- or R276-infected cells contained, in addition, several much longer RNAs that reacted with the gene VIII probe (lanes a,b), in agreement with the data of Smits et al. (1980), Cashman et al. (1980), and La Farina and Model (1983).

Adventitious -35 region

The presence of the 370-nucleotide RNA in pJB1- and pJB2-containing cells and the failure of the lac repressor overproducing host

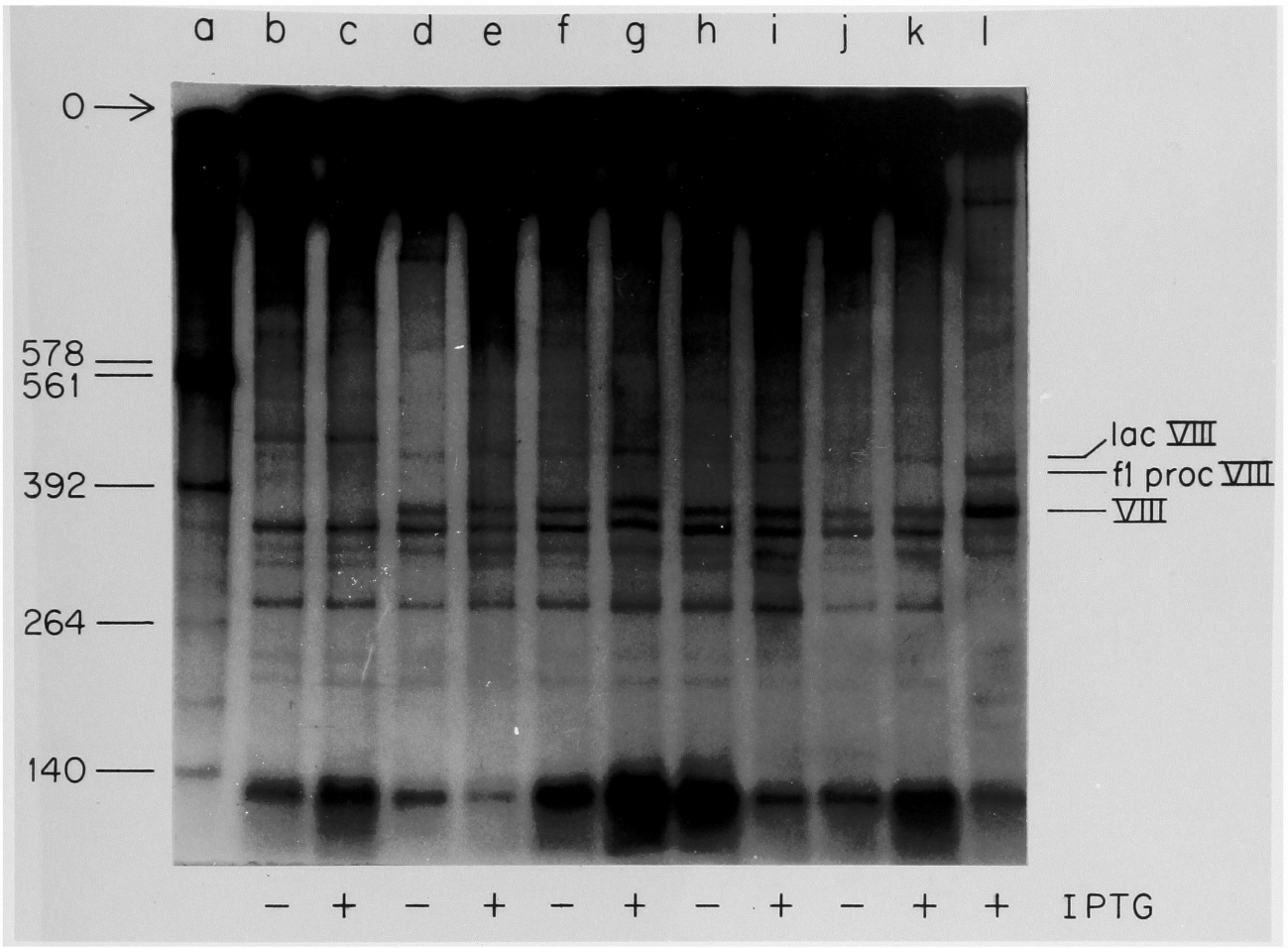


Figure 5.2. pJB1 and pJB2 specify a lac-inducible RNA. Cells were grown in peptone without (-) or with (+) 10^{-3} M IPTG as inducer, labeled with ^{32}P-phosphate, and the RNA extracted and electrophoresed on a 4% polyacrylamide gel in TBE buffer as described in Chapter 2. The host strains and plasmids were: K38.pBR322 (-)b, (+)c; K38.pJB1 (-)d, (+)e; K38.pJB2 (-)f, (+)g; K561.pJB1 (-)h, (+)i; K561.pJB2 (-)j, (+)k; K561 infected with R276 (+)l. The positions of the lacVIII, processed f1 VIII, and gene VIII RNAs are shown on the right. Lane a contains the RNAse III cleavage products of T7 DNA transcribed in vitro (kindly provided by H. Frankfort) as size markers; sizes in nucleotides are indicated at the left (Dunn and Studier, 1981). O marks the gel origin.

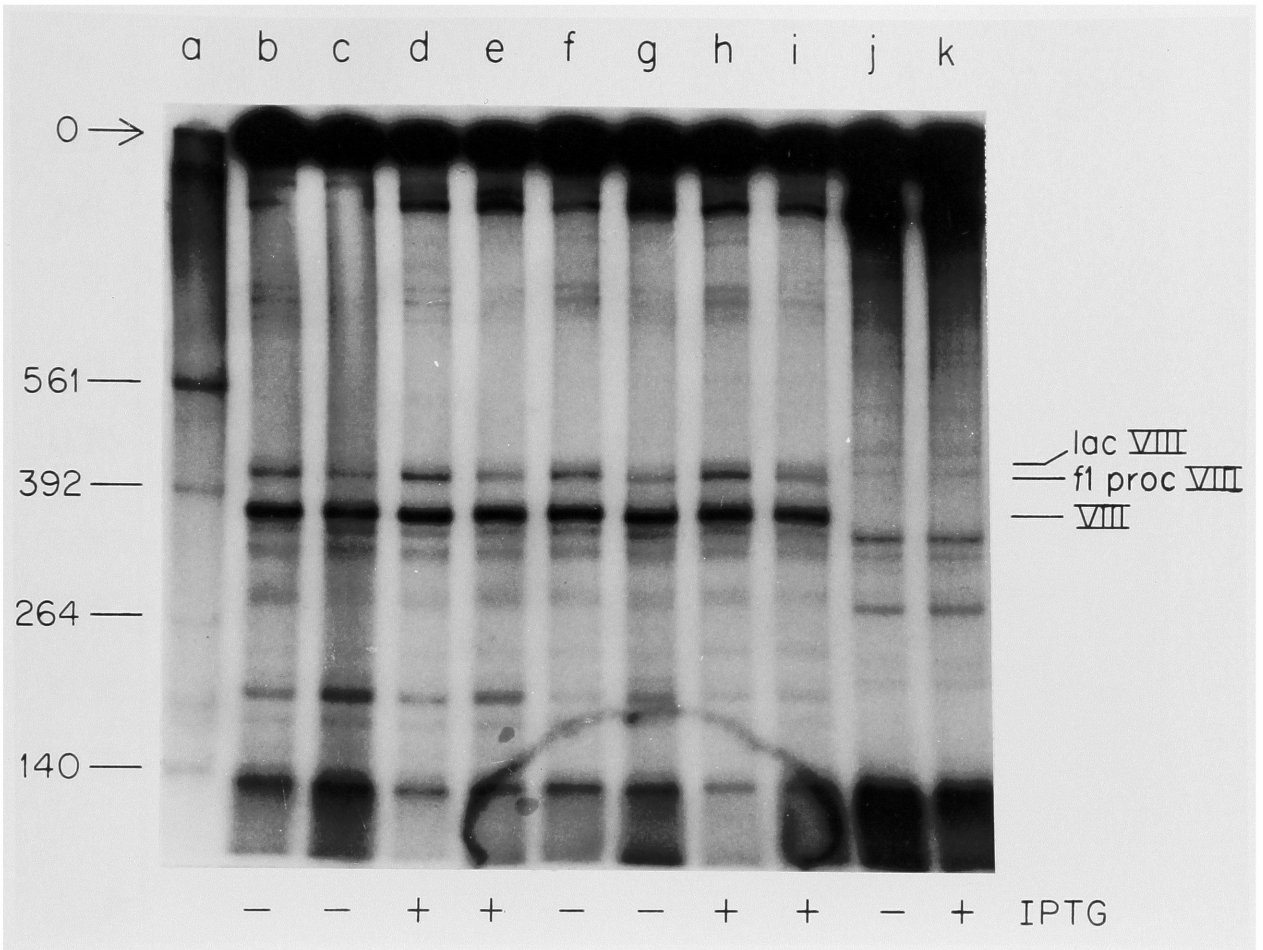


Figure 5.3. R276 specifies a lac-inducible RNA. Cells were grown in peptone without (-) or with (+) 10^{-3} M IPTG as inducer, infected with R276 or wild type f1 phage, labeled with ^{32}P-phosphate, and RNA was extracted and electrophoresed on a 4% polyacrylamide gel in TBE buffer as described in Chapter 2. Phage and host strains were: f1/K38 (-)b, f1/K561 (-)c, f1/K38 (+)d, f1/K561 (+)e, R276/K38 (-)f, R276/K561 (-)g, R276/K38 (+)h, R276/K561 (+)i, uninfected K561 (-)j, uninfected K561 (+)k. The positions of the lacVIII, processed f1 VIII, and gene VIII RNAs are shown on the right. T7 RNA size markers in lane a are described in the legend to Figure 5.2.

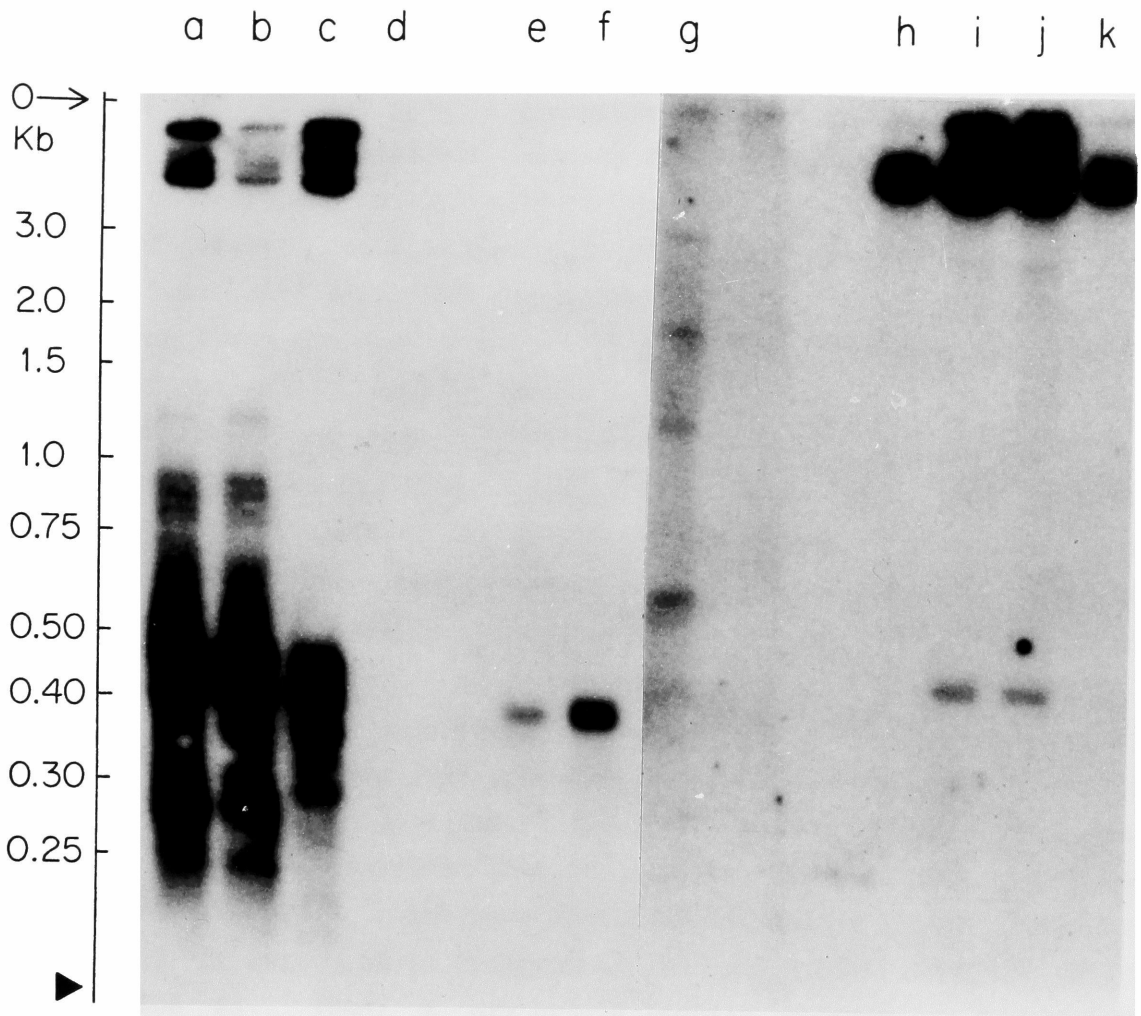


Figure 5.4. Northern hybridization of lac- and gene VIII-containing RNAs. Unlabeled RNA extracted from K38 infected with R276 (lanes b,i) or wild type f1 (a,h) and K38 harboring pJB2 (c,j) or pBR322 (d,k) was glyoxalated, electrophoresed on a 2.5% agarose gel in 10 mM phosphate buffer (pH 7.1), transferred to nitrocellulose, and hybridized (Branch et al., 1981) to 3'end-labeled probes prepared as described in Chapter 2. Lanes a-d were hybridized to a 128 bp MboI/TaqI fragment of f1 RF spanning positions 1382-1510 (internal to gene VIII); lanes h-k to the 95 bp EcoRI/PvuII lacUV5 operator-promoter fragment from pGL101 (Guarente et al., 1980). Washing of the filters was carried out at 73° (gene VIII probe) or 65° (lacOP probe), since the postulated, short 36 bp lacOP probe/lacVIII mRNA hybrid was calculated to have a Tm of ~75° (Davis et al., 1980), and indeed was not detected when washed at 73°. Exposure was at -70° for 4 days (lanes a-f) or 3 weeks (lanes g-k). Hybridization near the gel origin (0) is due to phage, plasmid, and host DNA. Labeled RNA size markers (kindly provided by A. Branch) were glyoxalated, electrophoresed, and transferred from the same gel: (e) 359-nucleotide linear PSTV (Gross et al., 1980), (f) 371-nucleotide linear and circular CEV (Visvader et al., 1982), (g) 264, 392, 561, 578, 1140, 1670, and 2749 nucleotide RNAse III cleavage products of T7 DNA transcribed in vitro (Dunn and Studier, 1981). A scale in kb calculated from the T7 markers is shown on the left.

K561 to shut off coat protein synthesis completely were at first puzzling, since a cp insert missing f1 nucleotides 1095-1177 could not direct coat protein synthesis in the phages R273 and R274 (Chapter 4). This cp insert was also cloned in pBR322 to give pJB84 and pJB85 (see Figure 5.6, lines 3-6). The 370-nucleotide RNA was not produced in K38 harboring these plasmids (Figure 5.5, lanes e,f). In contrast, pJB81, a clone of the am8H1 allele cp insert which contains nucleotides 1095-1924, obtained from phage R267 (Chapter 4), produced a large quantity of 370-nucleotide RNA in K38 cells (Figure 5.5, lane d). [The analogous wild type cp insert from R269 cannot be cloned in pBR322 due to the lethal effect of high levels of coat protein expressed from the gene VIII transcription unit in uninfected cells (Boeke, 1982). The approximately tenfold lower level of coat protein expressed from the lacVIII tu in pJB1 and pJB2 is not lethal.] Cleavage at position 1178 separates the Pribnow box of the gene VIII promoter from its -35 region. Inspection of the DNA sequence surrounding the fusion of the 95 bp lacOP fragment to f1 position 1178 revealed that the cloning manipulation had placed lac mRNA sequences which were fortuitously similar to the prokaryotic consensus -35 region 35 bp upstream of the gene VIII Pribnow box (Figure 5.6, line 2). Thus, a new hybrid lacVIII promoter was formed. The 370-nucleotide RNA in pJB1- and pJB2-containing cells is probably initiated at this hybrid promoter, but probably is identical to authentic gene VIII mRNA in sequence. This RNA is not under lac control, and therefore coat protein synthesis cannot be completely repressed in K561 (i^Q) cells.

The lacVIII tu, obtained as an EcoRI fragment purified from R276 RF, directed the synthesis of 420- and 370-nucleotide RNAs in an in vitro transcription reaction. The HpaII-H-C fusion fragment from R274 (-35 region deletion; see Figure 5.6, line 3) directed the synthesis of very little 370-nucleotide RNA compared to the analogous wild type HpaII-H-C (undeleted) fragment from R272 (data not shown).

Structural decay of the lacVIII mRNA

Far less 420-nucleotide lacVIII mRNA than 370-nucleotide gene VIII

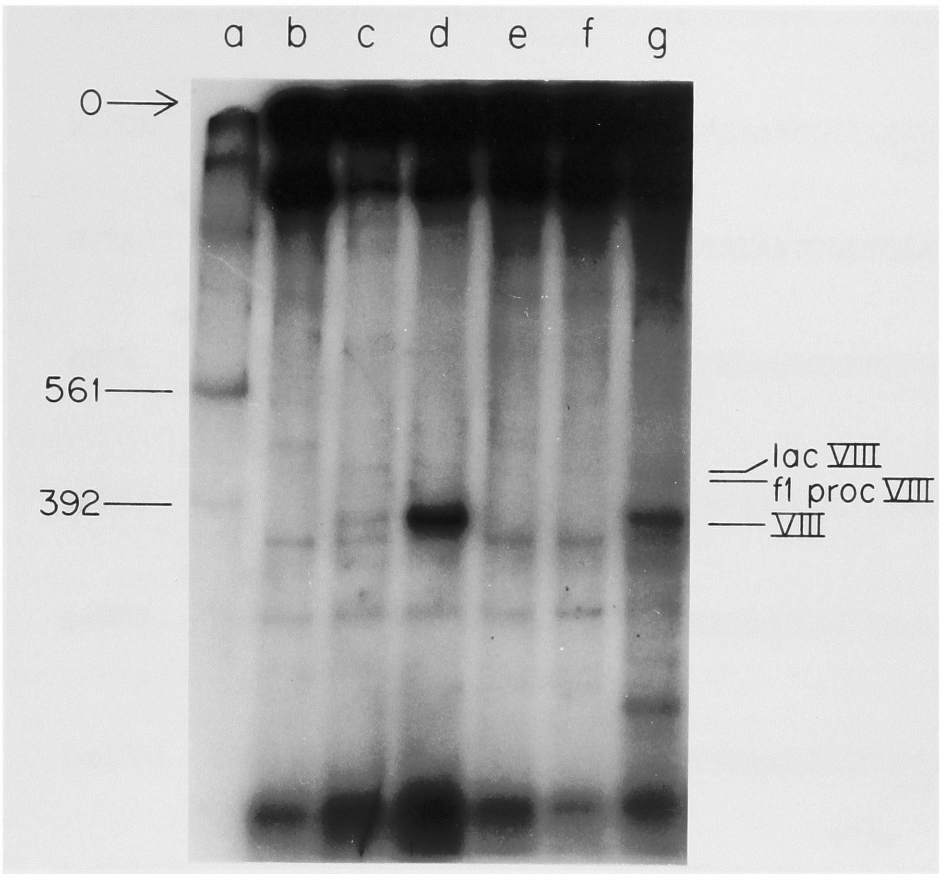


Figure 5.5. Gene VIII transcription unit with deletion of -35 region does not specify gene VIII RNA in vivo. Growth of cells in peptone, ^{32}P-phosphate labeling, RNA extraction, and electrophoresis on a 4% polyacrylamide gel in TBE buffer were performed as described in Chapter 2. Strains were: lane (b) K38.pBR322, (c) K38.pJB2, (d) K38.pJB81, (e) K38.pJB84, (f) K38.pJB85, (g) K38 infected with wild type f1. Lane a contains T7 RNA markers as described for Figure 5.2. The positions of the lacVIII, processed f1 VIII, and gene VIII RNAs are shown on the right.

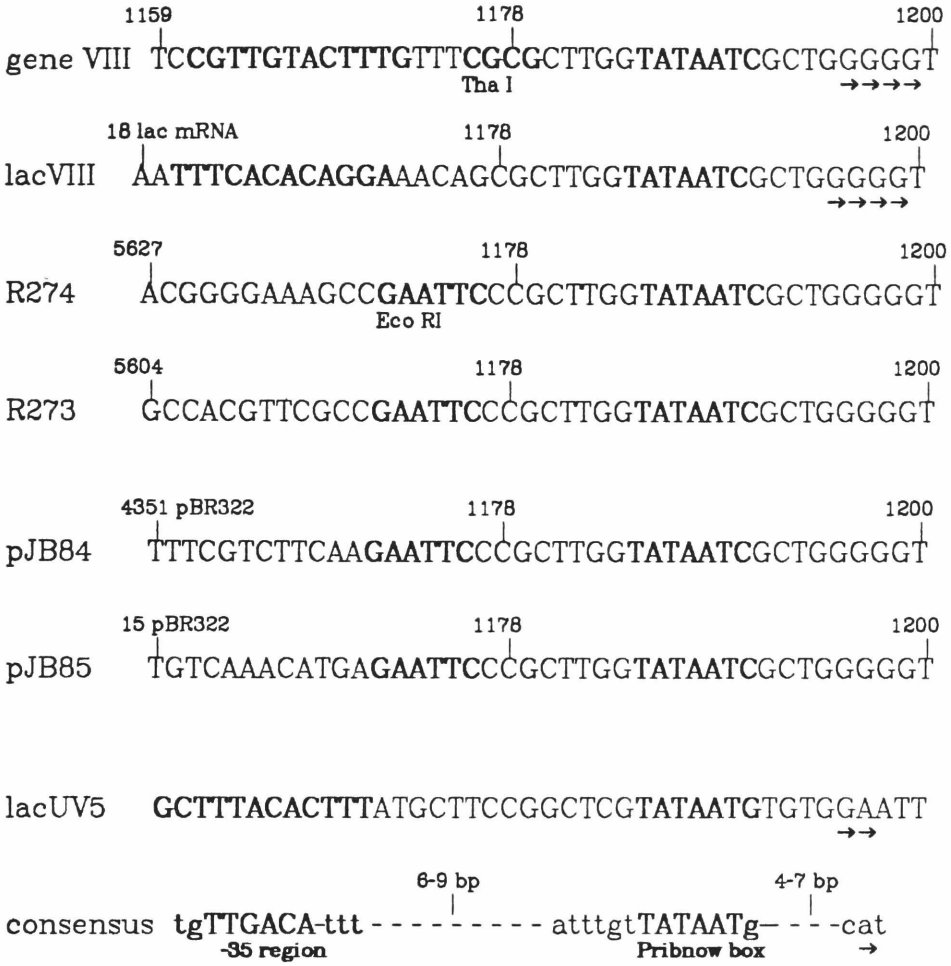


Figure 5.6. DNA sequences at -35 regions of active and inactive gene VIII promoters. Nucleotides homologous to the consensus -35 region and Pribnow box of prokaryotic promoters (line 8; Rosenberg and Court, 1979) are in bold face. Numbering of positions refers to f1 sequence unless otherwise noted (i.e., lac mRNA, pBR322). The ThaI site at position 1176 separating the -35 region and Pribnow box of the f1 gene VIII promoter (line 1) is in bold face, as are the EcoRI sites at the junctions of the -35 region deletion cp inserts in R274, R273, pJB84, and pJB85 (lines 3-6). The sequences upstream of the gene VIII Pribnow box in these constructions have little homology to the consensus -35 region. These hybrid promoters are inactive, as indicated by lack of small initiation arrows. The hybrid lacVIII promoter (line 2) has sequences in its -35 region which derive from positions 18-36 of lac mRNA and which reconstitute an active gene VIII promoter. These are homologous to the consensus -35 sequence. The E. coli lacUV5 promoter (line 7) is shown for comparison. The sequence shown for R274 was determined by the dideoxy terminator technique of Sanger et al. (1977); the lacVIII, R273, pJB84, and pJB85 sequences are predicted, based on the method of construction and the published sequences for lac (Reznikoff and Abelson, 1978), f1 (Hill and Petersen, 1982), and pBR322 (Sutcliffe, 1979).

mRNA was seen in vivo (Figures 5.2, 5.3, and 5.4). This could be explained by: 1) inefficiency of the lacUV5 promoter compared to the wild type gene VIII promoter or the fortuitous hybrid promoter on the lacVIII tu, and/or 2) instability of the 420-nucleotide RNA compared to the 370-nucleotide RNA. The half life of the 370-nucleotide gene VIII transcript is about 10 min (La Farina and Model, 1978; Rivera et al., 1978), while that of lac operon mRNA is about 1.5 min (Lim and Kennell, 1979).

I investigated the relative stabilities of the 420- and 370-nucleotide mRNAs by pre-labeling cells with ^{32}P-phosphate as before and then adding rifampicin to block initiation of transcription. Samples were removed just prior to, 2 min after, and 10 min after rifampicin addition, and the RNA was extracted immediately. The 370-nucleotide f1 gene VIII mRNA was quite stable under these conditions, with an approximate half life of 10 min (Figure 5.7, lanes b,c,d). The 370-nucleotide gene VIII mRNA from the fortuitous hybrid promoter on the lacVIII tu in pJB2 was similarly stable (lanes e,f,g). In contrast, most of the 420-nucleotide lacVIII mRNA had decayed by 2 min after rifampicin addition, and by 10 min this mRNA had disappeared entirely (lanes e,f,g). Unlike the lacVIII mRNA, the slightly smaller processed f1 gene VIII mRNA appeared to be stable (lanes b,c,d).

Discussion

Attachment of the 5' 36 nucleotides of the lac operon mRNA at the 5' end of the 369-nucleotide gene VIII mRNA appears to drastically reduce the stability of the gene VIII mRNA. The hybrid lacVIII mRNA decays with the lac half life of ~1.5 min, as opposed to the gene VIII half life of ~10 min. This result suggests that the 5'end of these mRNAs has an important role in determining stability. It is not yet known whether the 420-nucleotide lacVIII mRNA is completely degraded, or whether it decays to a smaller, stable species. One possibility is that it is processed to yield a 370-nucleotide gene VIII mRNA. Likewise, the 2000-nucleotide gene II mRNA, which is 3' coterminal with the gene VIII mRNA, is very unstable (Cashman et al., 1980; La Farina and Model,

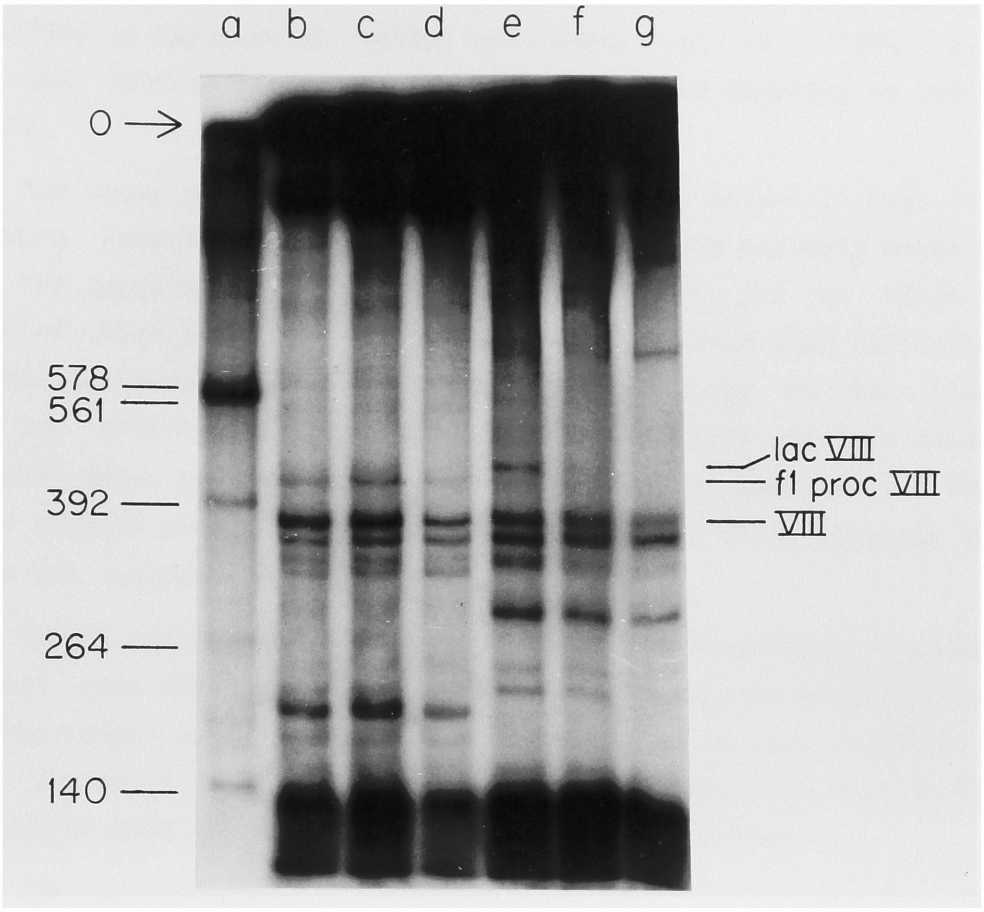


Figure 5.7. Decay of the lacVIII RNA. K38 cells were grown in peptone to log phase, infected with f1, and labeled from 15-45 min post-infection with ^{32}P-phosphate. K38.pJB2 were grown to the same density in peptone plus 10^{-3} M IPTG, and then labeled for 30 min. At the end of the labeling periods, rifampicin was added to 200 ug/ml final concentration. Samples were removed just prior to, 2 min after, and 10 min after rifampicin addition, and the RNA was extracted immediately. Conditions for RNA labeling, extraction and electrophoresis on a 4% polyacrylamide gel were as described in Chapter 2. T7 RNA size markers in lane a are as described for Figure 5.2. Samples are: f1-infected K38 just prior to (b), 2 min after (c), and 10 min after (d) rifampicin addition; K38.pJB2 just prior to (e), 2 min after (f), and 10 min after (g) rifampicin addition. The positions of the lacVIII, processed f1 VIII, and gene VIII RNAs are shown on the right.

1983). The accumulation of the processed f1 gene VIII mRNA species identified by Smits et al. (1980) and Cashman et al. (1980) suggests that the 2000-nucleotide gene II mRNA could be decaying to yield this species.

The decay phenomenon might be explained by either or both of the following considerations. 1) The 5'end sequence directly mediates decay; for example, by providing a recognition site for an RNAse. The extent of translation of the 5'end of the mRNA could also influence susceptibility to degradation. 2) The altered sequence at the 5'end in some way prevents the functioning of a stabilizing sequence within the gene VIII mRNA, perhaps by altering the secondary structure. Translational effects could conceivably play a role here also, although the lac AUG is not included in the hybrid lacVIII mRNA.

The 18 internal gene VII nucleotides that presumably connect the lac and gene VIII mRNA sections are normally present on the processed, slightly longer gene VIII mRNA which accumulates in vivo (Smits et al., 1980; Cashman et al., 1980). Thus, it is unlikely that this portion of the lacVIII mRNA is responsible for its destabilization.

The -35 "recognition" sequence upstream of the Pribnow box (Rosenberg and Court, 1979) appears to be essential for gene VIII promoter function both in vivo and in vitro. Replacement at the correct position with DNA homologous to the consensus sequence generated a weaker promoter, while replacement with nonhomologous DNA destroyed promoter function. Okamoto et al. (1977) had obtained data suggesting that the -35 region was not required for gene VIII promoter function in vitro, a result at variance with the data for most other prokaryotic promoters and with my results. Their results might be explained by the reaction conditions they employed. In f1 as in other prokaryotic genomes, -35 sequences are important for promoter activity. The -35 region of the gene III promoter likewise seems to be essential for promoter function in vivo. Furthermore, a Pribnow box sequence lacking a -35 region, which is present on the - strand of f1 at position 5357, is not active as a promoter in vitro (data not shown).

Chapter 6

A Rho-dependent Transcription Termination Signal in Bacteriophage f1

Introduction

Transcription termination in the filamentous bacteriophage f1 (fd, M13) has been shown to occur at a site immediately following gene VIII in vitro (Edens et al., 1975; Sugimoto et al., 1977) and in vivo (Rivera et al., 1978; Cashman et al., 1980). Transcripts formed in vitro in the absence of the E. coli termination factor rho (Roberts, 1969) range in size from the 369 nucleotide gene VIII transcript to genome length RNAs (Okamoto et al., 1975; Seeburg and Schaller, 1975; Edens et al., 1975; Chan et al., 1975). Addition of purified rho protein to an in vitro transcription system reduced the sizes of the RNAs made, but the termination sites have not been characterized (Takanami et al., 1970; 1971).

The longest transcripts seen in vivo are about 4000 nucleotides and do not contain gene VIII coding sequences; rather, they are contained within the infrequently transcribed (IF) region of the genome shown in Figures 1.1 and 6.1a (Smits et al., 1980; LaFarina and Model, 1983).

The longest transcripts from the frequently transcribed (F) region are about 2000 nucleotides and contain gene VIII sequences near their 3'termini (Cashman and Webster, 1979; Cashman et al., 1980; Smits et al., 1980; LaFarina and Model, 1983). The IF and F regions are separated by the 505 bp intergenic region (IG) which contains the origins of + and - strand DNA replication (Tabak et al., 1974; Horiuchi and Zinder, 1976; Suggs and Ray, 1977) but lacks protein coding sequences (Vovis et al., 1975; van den Hondel et al., 1976). Very few if any transcripts cross the IG (Smits et al., 1980; LaFarina and Model, 1983). Since transcripts made in vitro in the absence of rho protein do cross the IG, it seemed possible that a rho-dependent terminator located in the IG might act to prevent transcription through this region in vivo.

Rho-dependent terminators which have been characterized are λt_{R1} (Rosenberg et al., 1978; Court et al., 1980), λt_{RO} (Calva and Burgess, 1980), the E. coli $tRNA^{Tyr}$ terminator (Küpper et al., 1978), and *trp t'*

(Platt, 1981; Wu et al., 1981). These terminators all occur in A-T rich regions. Except for trp t', the actual sites of termination are preceeded by the common sequence (CA)ATCAA. A region of dyad symmetry also preceeds the termination sites in λt_{R1} and λt_{RO}. However, none of the transcripts terminated at these sites end in runs of U's, in contrast to RNAs terminated at rho-independent terminators (Adhya and Gottesman, 1978; Rosenberg and Court, 1979). The transcripts terminated in vitro in the presence of rho protein have heterogeneous 3'ends scattered across 2-5 (λt_{R1}, λt_{RO}, $tRNA^{Tyr}$) or 50 (trp t') nucleotides.

Since the mechanism by which rho causes transcription termination is still obscure, I sought to characterize a new rho-dependent termination signal in order to discover more about the nature of rho action in vivo. In the work presented here, an f1 rho-dependent terminator was identified by cloning the gene IV-IG boundary region in a plasmid vector in which termination signals inserted between the galactose promoter (Pgal) and the galactokinase gene (galK) prevent expression of galactokinase on the plasmid (McKenney et al., 1981). Then, termination was studied in this region of the normal f1 phage genome during infection of rho^+ and rho^- hosts, using Northern hybridization analysis and S1 nuclease mapping methods.

Results

Identification of a rho-dependent transcription termination signal distal to gene IV

The HgaI/HpaII 469 restriction fragment spanning 350 bp of the f1 gene IV carboxyl terminus and 119 bp of the IG (Figure 6.1b) was cloned in the plasmid vector pKG1900 of McKenney et al. (1981) between the E. coli galactose promoter (Pgal) and the structural gene for the enzyme galactokinase (galK) (see Figure 6.2). Complementation of a galK missense mutation in host strain K702 (N100 $galE^+T^+K^-$, $recA^-$) was used to score terminator phenotypes on MacConkey galactose ampicillin indicator plates. Clones containing a functional terminator prevent transcription of galK on the plasmid and give white colonies on MacGal plates, while the vector pKG1900 and clones which do not contain a func-

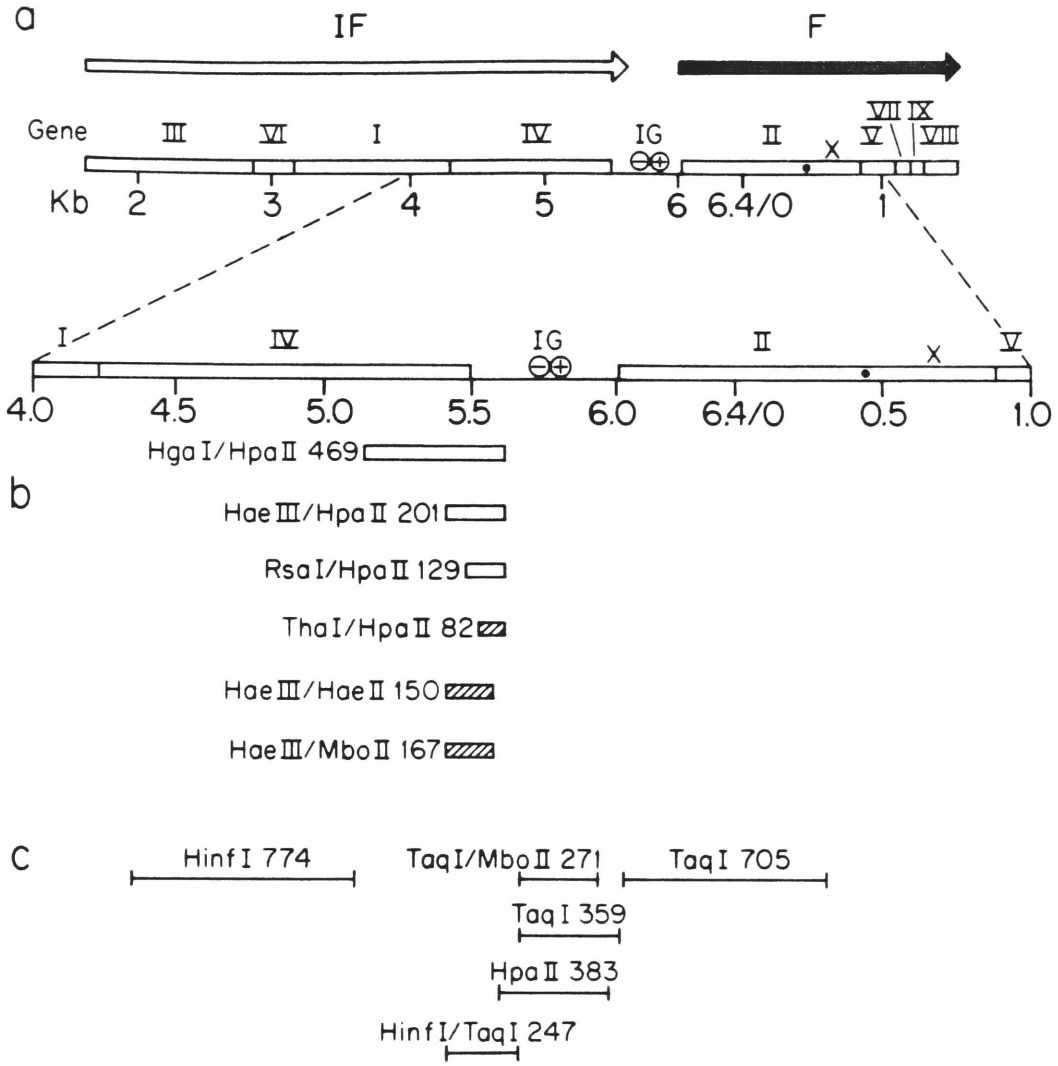


Figure 6.1. Map showing f1 genome, terminator plasmid clones, and hybridization probes. a. Map of bacteriophage f1, shown opened between genes VIII and III. Genes are denoted by Roman numerals, nucleotide positions in kb are from the unique HincII site. IG is the intergenic region containing the origins of - and + strand DNA synthesis and the rho-dependent transcription termination signal. IF and F denote the infrequently and frequently transcribed regions of the genome, respectively (LaFarina and Model, 1983). A rho-independent termination signal exists between genes VIII and III (Edens et al., 1975; Rivera et al., 1978). The region surrounding the IG is shown expanded below the complete map. b. f1 DNA fragments cloned in terminator plasmid vectors are aligned below the expanded f1 map. Names of fragments refer to restriction enzymes used to generate 5'/3'ends and sizes of fragments in bp. Open bars indicate fragments active as terminators, hatched bars indicate inactive fragments. c. f1 DNA fragments used for 3'end labeled hybridization probes are aligned with the expanded f1 map. The three fragments on the top line were used in the Northern hybridization experiment shown in Figure 6.3, the three fragments below in S1 nuclease mapping experiments.

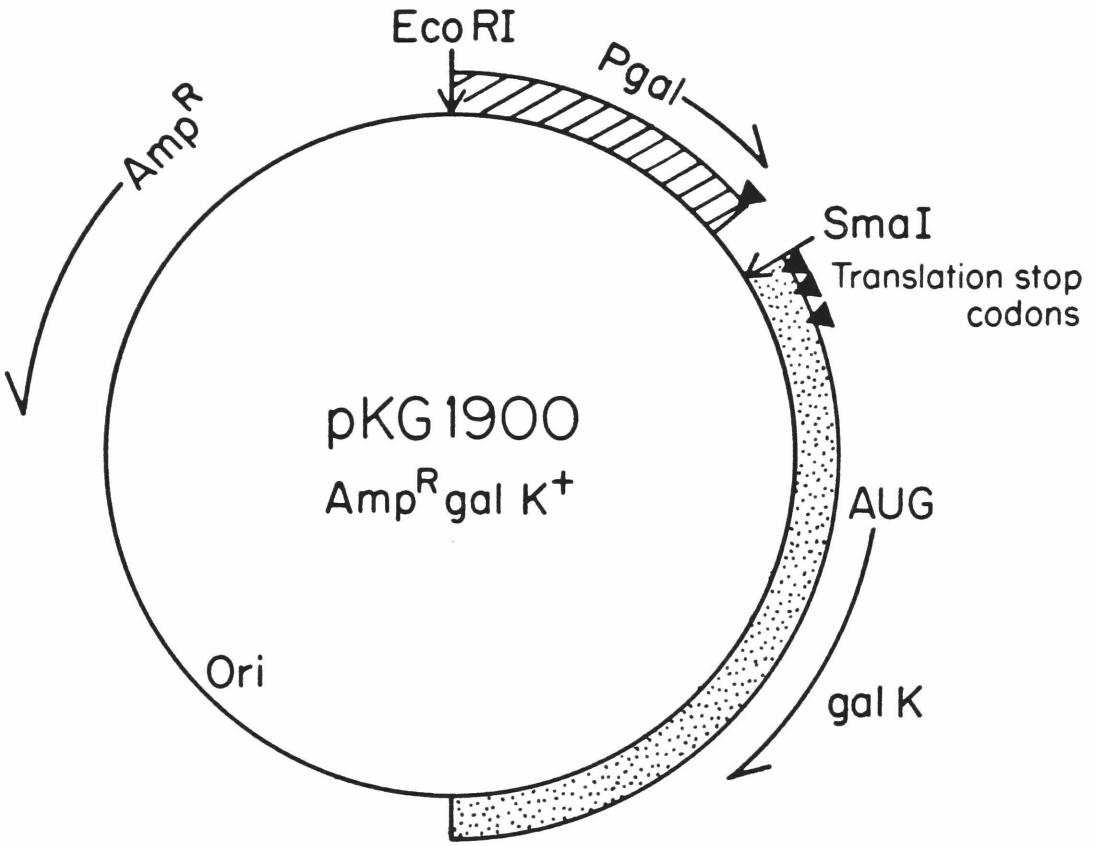


Figure 6.2. The pKG1900 plasmid assay for transcription terminators, developed by McKenney et al. (1981). Plasmid pKG1900 contains the E. coli galactose promoter (Pgal) and the structural gene for the enzyme galactokinase (galK), with a SmaI site for cloning located between them. In pKG1900 transcription of galK from the gal promoter causes expression of the enzyme, which can complement a galK host mutation. Insertion of a functional termination signal at the cloning site prevents transcription of galK, so complementation is abolished. MacConkey galactose indicator plates provide a simple test for complementation by the plasmid in a galK mutant host: complementers give red colonies, non-complementers are white. Thus a host harboring pKG1900 which expresses galactokinase is red, while clones with a functional terminator insert are white.

Table 6.1. Terminator Activity of Gene IV-IG Boundary Fragments In Vivo

Terminator fragment	Orient-ation	Vector	Host	MacGal assay	S1 nuclease mapping assay 3'-end 5565
HgaI/HpaII 469	+	pKG1900	K702(rho$^+$)	w	y
"	-	"	"	r	
"	+	"	K791(rho$^+$)	w	y
"	+	"	K793(rho$^-$)	r	n
HaeIII/HpaII 201	+	pKG100	K702(rho$^+$)	w	y
"	-	"	"	r	
RsaI/HpaII 129	+	"	"	w	y
"	-	"	"	r	
ThaI/HpaII 82	+	"	"	r	n
"	-	"	"	r	
HaeIII/HaeII 150	+	"	"	r	n
"	-	"	"	r	
HaeIII/MboII 167	+	"	"	r	n
"	-	"	"	r	
None		pKG1900	"	r	
None		pKG100	"	r	

Locations of terminator fragment endpoints on the f1 genome are shown in Fig. 6.1. Orientation + means Pgal-promoted transcription on the vector crosses the terminator fragment in the same direction as f1 transcription on the phage genome,so that transcripts contain viral (+) strand sequences; - means the inverse orientation. Vectors contain Pgal-cloning sites-galK, pBR322 origin of replication and ampicillin resistance gene (McKenney et al., 1981). K702 is (N100 galE$^+$T$^+$K$^-$, recA$^-$ rho$^+$), K791 is (C600 galE$^+$T$^+$K$^-$, recA$^-$, rho$^+$), and K793 is (C600 galE$^+$T$^+$K$^-$, recA$^-$, rho$^-$). w and r indicate white or red colonies at 37° on MacConkey galactose ampicillin plates, corresponding to terminator and non-terminator phenotype, respectively. S1 nuclease mapping was performed using a cognate DNA probe from each terminator plasmid clone 3'end labeled at the f1 HinfI 5439 site, or at the vector HinfI 463 site in the galE sequence for clones lacking the f1 HinfI site (RsaI/HpaII 129 and ThaI/HpaII 82). 3'end 5565 refers to f1 position 5565 within terminator fragments; y indicates the presence, n the absence of an RNA 3'end at this position in the clones.

tional terminator are red in this host (McKenney et al., 1981). The HgaI/HpaII 469 fragment inserted with the + orientation such that Pgal-promoted transcripts contain f1 viral (+) strand sequences (as do all f1 transcripts produced during infection) gave a white terminator phenotype, while the inverse (-) orientation gave a red non-terminator phenotype (Table 6.1, lines 1 and 2). Thus, this fragment is active as a terminator only in its naturally occurring orientation.

The nitA702ts allele, hereafter referred to simply as rho$^-$, has been characterized as a temperature sensitive rho mutation. The original rho mutant strain is inviable at high temperature, but allows transcription of λ DNA independent of the N gene even at low temperatures. The purified mutant rho protein has altered properties in vitro (Inoko et al., 1977; Imai and Shigesada, 1978; Shigesada and Imai, 1978). Strains K793 (galK$^-$, recA$^-$, rho$^-$) and K791 (galK$^-$, recA$^-$, rho$^+$) were constructed from galK missense strain K701 (C600 galE$^+$T$^+$K$^-$) by P1 transduction and used as hosts to test terminator function by the MacGal assay. The terminator phenotype of clone HgaI/HpaII 469+ was abolished in rho$^-$ strain K793 (Table 6.1, line 4).

Subclones of the HgaI/HpaII 469 terminator fragment were constructed in the plasmid vector pKG100 (pKG1900 with multiple cloning sites between Pgal and galK; K. McKenney, personal communication), and tested for terminator activity in the MacGal plate assay (Figure 6.1b; Table 6.1, lines 5-14). The smallest fragment tested which still had terminator activity, RsaI/HpaII 129 (line 7), contained the last 10 nucleotides of gene IV and 119 nucleotides of the IG. Fragment ThaI/HpaII 82, whose 5'end is 35 bp distal to the gene IV UAG, did not have terminator activity (line 9). Fragment HaeIII/MboII 167, whose 3'end is 79 bp distal to the gene IV UAG, did not have terminator activity (line 13). Thus a region of ~100 nucleotides distal to gene IV is necessary for transcription termination in the pKG1900 assay system.

Detection of longer f1 RNA species containing IG sequences in a rho$^-$ host

RNA extracted at 20 min postinfection from f1 infected K819 (rho$^-$) and K38 (rho$^+$) hosts was glyoxalated, electrophoresed on a 1.2% agarose gel, transferred to nitrocellulose, and hybridized (Alwine et al., 1977 as modified by Branch et al., 1981) to 3'end labeled DNA probes (Figure 6.1c) containing f1 sequences internal to gene IV (HinfI 774), distal to gene IV but internal to the IG (TaqI/MboII 271), or farther downstream from gene IV and internal to gene II (TaqI 705). Figure 6.3 shows the results of this experiment. The pattern of f1 RNAs detected with the gene IV probe is altered in the rho$^-$ (lane e) as compared to the rho$^+$ host (lane d). There are new prominent species of ~1800 nucleotides present in the rho$^-$ host which are not present in the rho$^+$ host, in which the most prominent species are ~1600 nucleotides. The amount of RNA hybridizing to the gene IV probe is increased in the rho$^-$ host, particularly the larger species between 1800 and 4200 nucleotides. The IG probe reacts principally with the ~1800 nucleotide RNAs detected only in the rho$^-$ host (lane g). The ~1600 nucleotide species detected with the gene IV probe in the rho$^+$ host do not hybridize to the IG probe (lane f). In contrast, the RNA patterns observed using the gene II probe (which comes from the frequently transcribed region of the genome) are similar in the rho$^-$ and rho$^+$ strains, except for some shift towards the higher molecular weight species (1500-2000 nucleotides) in the rho$^-$ strain (lanes h,i).

The longest RNA species detected with the gene IV probe are ~4000 and ~4200 nucleotides in the rho$^+$ and rho$^-$ hosts, respectively (Figure 6.3, lanes d,e, tenfold longer exposure not shown). The ~4200 nucleotide species seen in the rho$^-$ host also react with the IG probe, but the ~4000 nucleotide species found in the rho$^+$ host do not (lanes f,g). The longest RNAs hybridizing to the gene II probe are ~2000 nucleotides in both rho$^+$ and rho$^-$ hosts (lanes h,i). Genome length transcripts (6400 nucleotides) were not visible in either the rho$^-$ or the rho$^+$ host in this experiment, even after tenfold longer exposure of the filter.

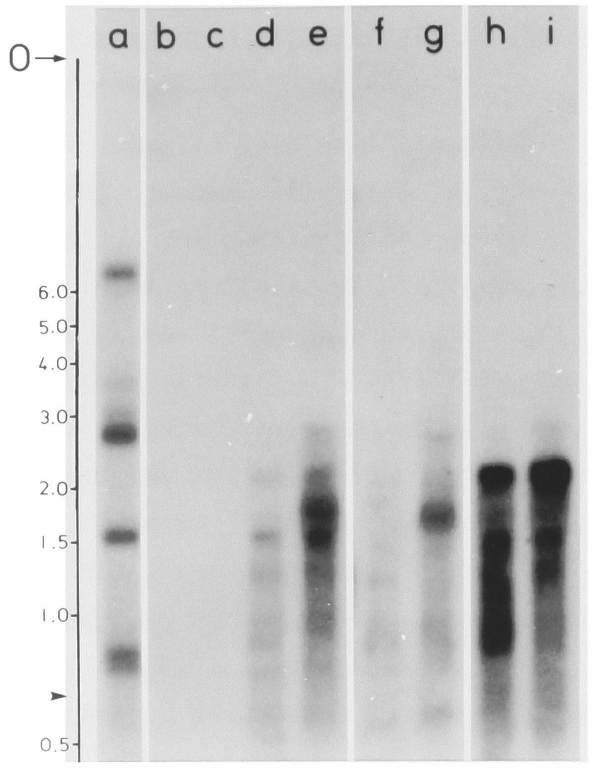


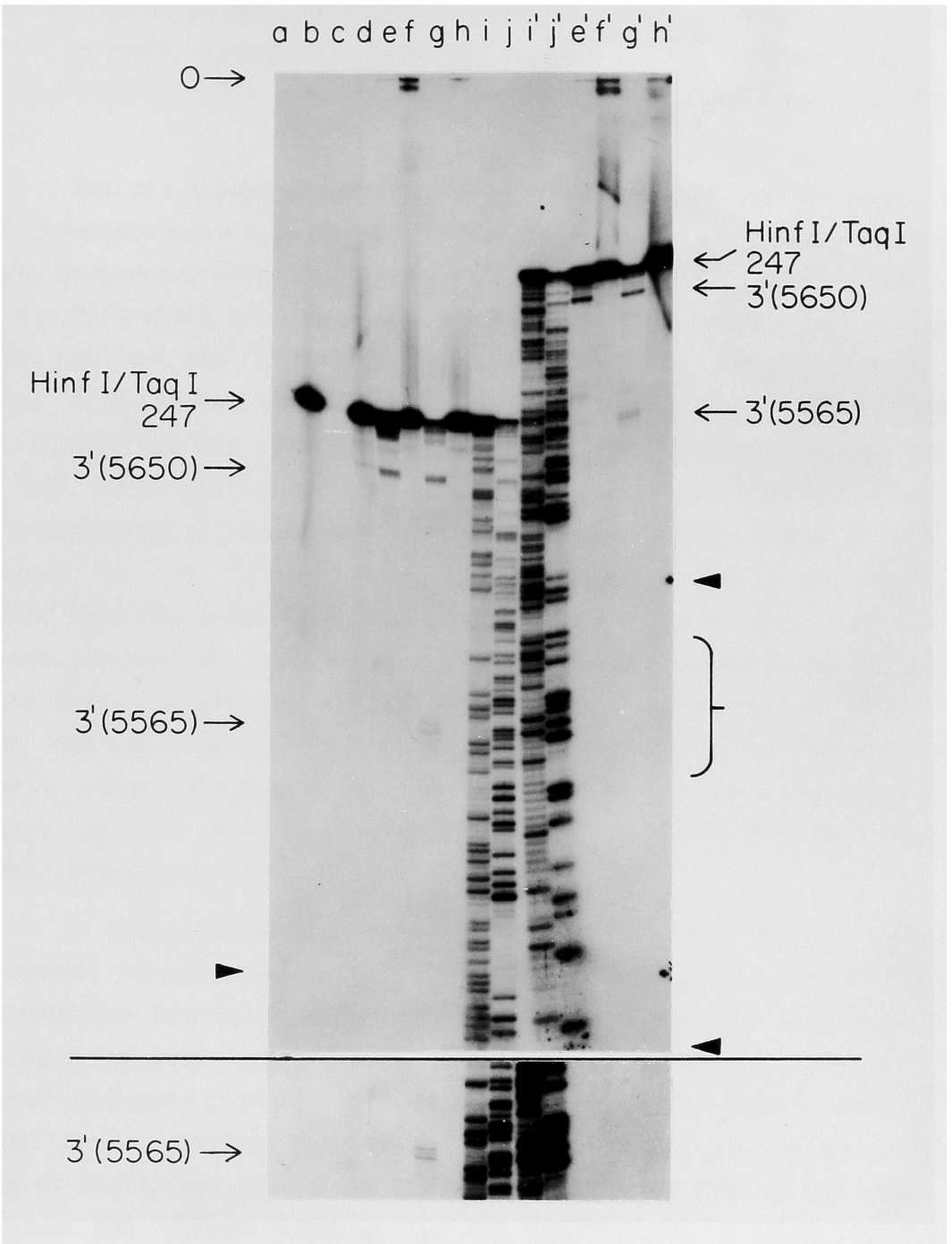
Figure 6.3. Detection of longer f1 RNAs hybridizing to the IG in a rho$^-$ host. RNA from f1 infected K38 (rho$^+$), K819 (rho$^-$), and uninfected K819 grown at 41.5^o was treated with pancreatic DNAse, glyoxalated, and electrophoresed on a 1.2% agarose gel in 10 mM phosphate buffer, pH 7.1 (Branch et al., 1981). Uniformly P^{32}-labeled f1 (R219) DNA size markers were glyoxalated and run in parallel. The nucleic acids were transferred to nitrocellulose and hybridized to 3'end labeled f1 DNA probes. Lane a) size markers: f1 viral strands and RF treated with HaeIII at 65^o. Single-stranded DNA is not cleaved under these conditions. The sizes of the bands in nucleotides are, from top to bottom, 6415 (genome length), 2685, 1623, 849, and 774 (a fusion fragment in R219 caused by conversion of HaeIII sites at f1 positions 5725 and 5867 to EcoRI sites by 4 base insertions; thus R219 is 8 bases longer than wild type f1; J. Boeke, unpublished). Scale at left is marked in kb, O shows gel origin, and ▶ indicates position of bromophenol blue dye marker. Lane b) RNAse treated f1 infected K819 RNA. Lane c) uninfected K819 RNA. Lanes e, g, i) f1 infected K819 (rho$^-$) RNA. Lanes d, f, h) f1 infected K38 (rho$^+$) RNA. Lanes b-e were hybridized to probe HinfI 774 (internal to gene IV), lanes f-g to probe TaqI/MboII 271 (distal to gene IV and internal to IG), and lanes h-i to probe TaqI 705 (farther downstream from gene IV and internal to gene II); see Figure 6.1c. Ten-fold longer exposure of the filter (not shown) revealed no bands in lanes b-c, no bands longer than the 4.2 kb band (faintly visible here) in lanes d-g, and no bands longer than 2 kb in lanes h-i.

S1 nuclease mapping of transcripts in the gene IV-IG boundary region reveals a rho-dependent 3'end

RNA from f1 infected K819 (rho$^-$) and K38 (rho$^+$) was hybridized to 3'end labeled DNA probes (Figure 6.1c) and digested with S1 nuclease (Aiba et al., 1981). The protected DNA products were electrophoresed on sequencing gels next to the same 3'end labeled DNA fragments which had been subjected to Maxam-Gilbert sequencing reactions (Maxam and Gilbert, 1980). Using probe HinfI/TaqI 247 labeled at f1 position 5439 in the carboxyl terminus of gene IV and extending 188 bp past the gene IV UAG into the IG, protected DNA bands of ~127 and ~212 nucleotides were seen in the rho$^+$ host, labeled 3'(5565) and 3'(5650) respectively (Figure 6.4, lanes g,g'; the lower portion of Figure 6.4 shows a darker print of the same autoradiogram, on which the 3'(5565) bands are more clearly visible). These bands correspond to RNA 3'ends at positions ~5565 and ~5650 in the IG (Figure 6.4, lanes i,j,i',j'). No protected DNA bands shorter than the ~127 nucleotide band 3'(5565) were observed. The ~127 nucleotide band was absent when f1 RNA from the rho$^-$ host was used, and the amount of protected DNA longer than ~220 nucleotides was greatly increased (Figure 6.4, lanes e,e'). The same results were obtained using RNA from cells infected at either 34^o or 41.5^o. Thus, in this as in the λ system (Inoko et al., 1977), the effect of the rho mutation nitA702ts is not temperature dependent. The high temperature could be tested in K819 since these cells are not very temperature sensitive for growth, and support f1 phage production at one-third the rate of the wild type host at high temperature (M. Russel, personal communication).

To confirm that the absence of transcription termination at position 5565 in the f1 gene IV-IG boundary region in host K819 was caused by the mutant rho allele, a plasmid clone of the wild type rho allele (pEG25, obtained from M. Gottesman) was introduced by transformation into K819 and K38, and S1 mapping of f1 RNA from these hosts was performed with probe HinfI/TaqI 247 as described above (data not shown). The 3'end at position 5565 was restored and protected DNA longer than ~220 nucleotides was eliminated in host K837 (K819.pEG25). Thus, the wild type rho clone complemented the rho mutation in K819. The mul-

Figure 6.4. S1 nuclease mapping of rho-dependent f1 RNA 3'end. 3'end labeled probe HinfI/TaqI 247 was hybridized to RNA from uninfectd K819 (lanes a,b), uninfected K38 (lanes c,d), f1 infected K819 (rho^-) (lanes e,f), and f1 infected K38 (rho^+) (lanes g,h). Samples in lanes a, c, e, and g were digested with S1 nuclease. The 3'end labeled probe was also subjected to the C+T (lane i) and G (lane j) sequencing reactions of Maxam and Gilbert (1980). Samples were electrophoresed on an 8% acrylamide, 7M urea gel in TBE buffer. O marks the origin. For lanes a-j the position of the xylene cyanol dye is shown at the bottom left (►). Electrophoresis was carried out for a shorter time on samples marked with primes; the middle and bottom right (◄) mark the positions of the xylene cyanol and bromophenol blue dyes respectively. The intact DNA probe and protected DNA fragments corresponding to RNA 3'ends at the indicated nucleotide positions are marked. The lower portion of the figure shows a darker print of the part of the autoradiogram bracketed in the upper portion. Protected DNA bands comigrating with sequencing reaction bands correspond to the previous nucleotide in the f1 sequence, since the Maxam-Gilbert reactions eliminate the modified terminal nucleotide but protection from S1 digestion retains it. RNA for this experiment was extracted from cells grown and infected at 34°, but identical results were obtained with cells grown and infected at 41.5° (not shown).


a b c d e f g h i j i' j' e' f' g' h'
0→
Hinf I/Taq I
247
3'(5650)→
3'(5565)→
Hinf I/Taq I
247
3'(5650)
3'(5565)
3'(5565)→

ticopy rho clone gave no effect beyond the normal function of the single copy genomic locus: S1 mapping results with hosts K38, K535 (K38.pBR322), K836 (K38.pEG25), and K837 (K819.pEG25) were all identical.

RNA was transcribed in vitro in the absence of rho protein, using as template wild type f1 RF and RF of the vector phage CGF3 (Collaborative Research), which contains an insertion of 13 nucleotides at position 5615 which creates a PstI site. The in vitro RNA, and in vivo RNA from rho^+ and rho^- hosts infected with f1 or CGF3, was used for S1 mapping with probe HinfI/TaqI 247 for f1 or the corresponding fragment HinfI/TaqI 260 for CGF3 (data not shown). The entire lengths of the probes were protected with in vitro RNA. No ~127 nucleotide band corresponding to rho-dependent 3'end 5565 was formed. Only a very low amount of the ~212 nucleotide band corresponding to rho-independent 3'end 5650 was seen. The length of this band in CGF3 was ~225 nucleotides, so that the CGF3 3'end mapped to the same sequence as at f1 position 5650. In vivo RNA from f1 and CGF3 gave rho-dependent 3'end 5565 and rho-independent 3'end 5650 in the amounts seen in previous experiments. Thus, rho-dependent 3'end 5565 is not seen in vitro in the absence of rho protein, while rho-independent 3'end 5650 is formed very inefficiently in vitro.

f1 position 5565 is contained on all the clones with a terminator fragment insert, but position 5650 is not contained on any of them. S1 mapping was therefore done on RNA extracted from strain K702 (rho^+) containing the terminator fragment clones, in order to correlate the terminator phenotype with the rho-dependent 3'end at f1 position 5565. Cognate probes obtained from the terminator plasmids were 3'end labeled at the f1 HinfI 5439 site or at the vector HinfI 463 site in the galE sequence for clones lacking the f1 HinfI site. A protected DNA band corresponding to 3'end 5565 was seen using RNA from terminator clones active in the MacGal assay (Table 6.1). A band corresponding to 3'end 5565 was not seen in clones which were not active as terminators in the MacGal assay. Furthermore, 3'end 5565 was not seen in clone HgaI/HpaII 469+ in the rho^- host K793 (line 4), which also correlates with this

clone's non-terminator phenotype in the MacGal assay in K793.

Gene VIII RNA ends at the previously described rho-independent terminator of f1 (Edens et al., 1975; Sugimoto et al., 1977; Rivera et al., 1978; Cashman et al., 1980). As a control experiment, S1 mapping was performed in the region surrounding this terminator. No differences in the protected DNA bands corresponding to gene VIII RNA terminating at nucleotide 1564 were detected in the rho^- versus the rho^+ host using probe HinfI 610 spanning f1 nucleotides 1404-2014 (data not shown).

Termination is not affected in nusA1 mutant host

The E. coli termination factor nusA is involved in transcription termination at several E. coli operons (Ward and Gottesman, 1981; Greenblatt et al., 1981). However, S1 mapping with probe HinfI/TaqI 247 of RNA from an f1 infected nusA1 mutant host (K831) revealed no alteration of the 3'ends detected at the f1 rho-dependent terminator.

Quantity of rho-dependent 3'end is unchanged in RNAse III^- host

Differential stability of RNAs with different 3'ends could account for the low amount of correct 3'end 5565 detected in the rho^+ host compared to the 10-20 fold excess of "readthrough RNA" seen in the rho^- host (Figure 6.4, lane g vs. e). I considered the possibility that the E. coli processing enzyme RNAse III might act at hairpin A shown in Figure 6.6 to cleave and thus destabilize the correctly terminated RNA. However, S1 mapping experiments with probe HinfI/TaqI 247 of f1 RNA from an RNAse III^- host (K829) showed no difference in the amount or distribution of 3'ends compared to the wild type host.

f1 transcripts in rho^- host end within downstream region of IG

In order to determine whether the longer f1 transcripts detected in the rho^- host went through the IG into gene II or stopped within the IG near the origin of DNA replication, probes HpaII 383 and TaqI 359 (Figure 6.1c) were used for S1 mapping as above (Figure 6.5). Very little DNA protected from S1 was seen when these probes were hybridized to RNA from f1 infected rho^+ cells (lane b). Several intense bands were detected with RNA from f1 infected rho^- cells (lane d). These 3'ends

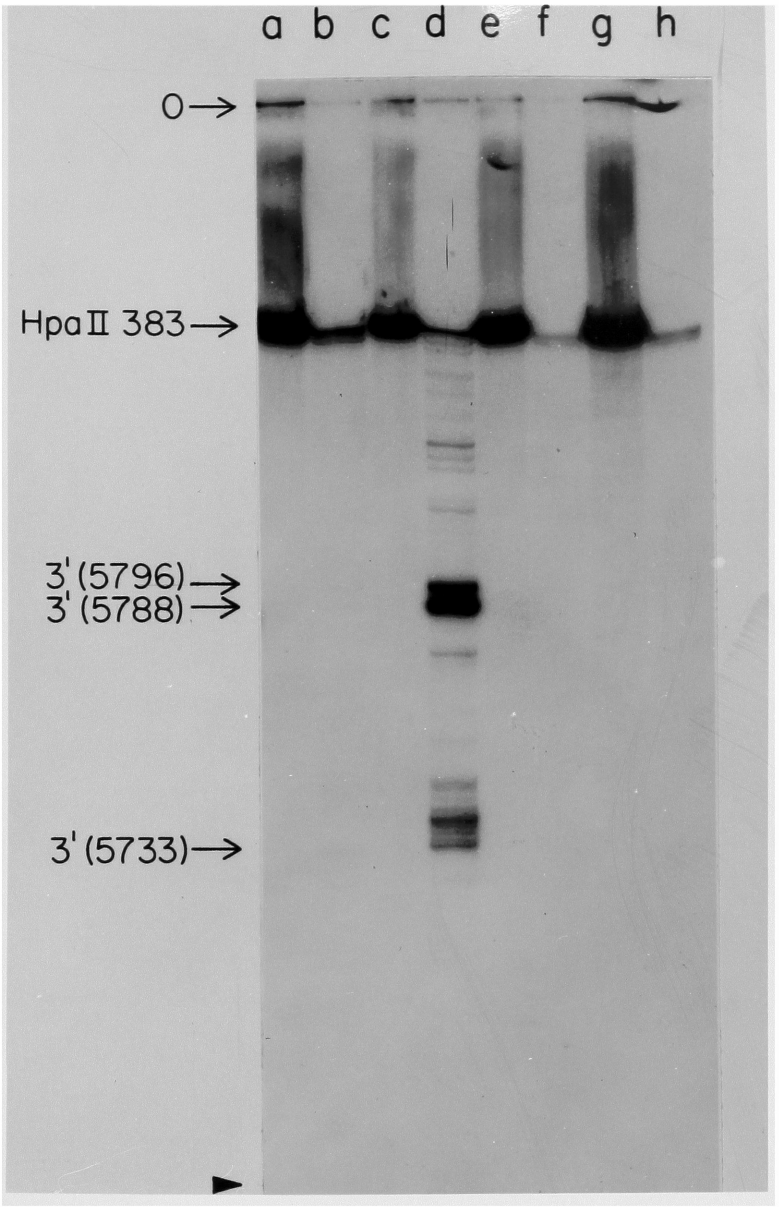


Figure 6.5. S1 nuclease mapping of f1 "readthrough RNA" in rho$^-$ host. 3'end labeled probe HpaII 383 was hybridized to RNA from f1 infected K38 (rho$^+$) (lanes a,b), f1 infected K819 (rho$^-$) (lanes c,d), uninfected K38 (lanes e,f), and uninfected K819 (lanes g,h). Samples in lanes b, d, f, and h were digested with S1 nuclease. Electrophoresis was on an 8% acrylamide, 7M urea gel in TBE buffer; O marks the origin, ► indicates the position of the xylene cyanol dye. The 3'end labeled probe was also subjected to the C+T and G Maxam-Gilbert sequencing reactions and electrophoresed in parallel (not shown) to identify the protected DNA bands in lane d. The intact DNA probe and protected DNA fragments corresponding to RNA 3'ends at the indicated nucleotide positions are marked. The ~15 adjacent bands beginning at position 5733 occur within the sequence of the ori-RNA primer for - strand DNA synthesis (see Figure 6.6). RNA for this experiment was extracted from cells grown and infected at 41.5°, but identical results were obtained with cells grown and infected at 34° (not shown). The prominent protected "readthrough" bands were also identified using probe TaqI 359 (not shown).

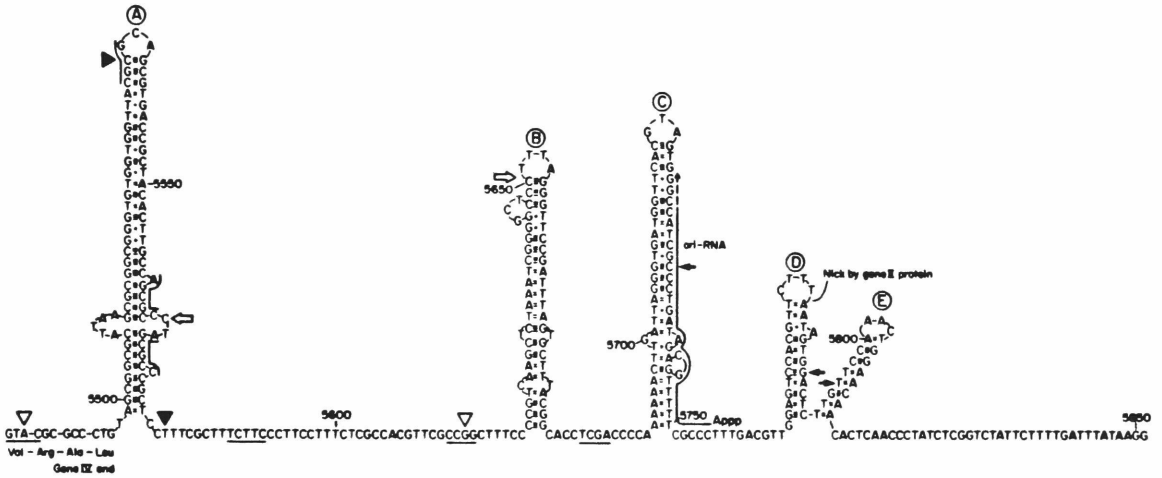


Figure 6.6. Sequence and structure of the f1 rho-dependent transcription termination signal. The f1 viral (+) strand sequence from positions 5486-5851 (Hill and Petersen, 1982) is drawn in potential secondary structures (Beck and Zink, 1981) distal to gene IV, and near the origins of - and + strand DNA synthesis (denoted by ori-RNA and nick by gene II protein, respectively). Restriction sites referred to in Figure 6.1 are underlined. The open triangles (▽) at positions 5488 and 5616 mark the 5' and 3' boundaries respectively of active terminator clone RsaI/HpaII 129+. The stippled triangles (▼) at positions 5535 and 5579 mark, respectively, the 5' boundary of inactive terminator clone ThaI/HpaII 82+ and the 3' boundary of inactive terminator clone HaeIII/MboII 167+. The open arrows (⇦) mark the rho-dependent RNA 3'end mapped to position 5565 and the rho-independent RNA 3'end mapped to position 5650. The solid arrows (⬅) mark the "readthrough RNA" 3'ends seen around positions 5733, 5788, and 5796 in f1 infected K819 (rho^-) hosts.

were mapped against HpaII 383 subjected to Maxam-Gilbert degradation: major 3'ends occurred at f1 positions ~5733, ~5788, and ~5796, which are within the IG near the origin of DNA replication (Figure 6.6).

Discussion

The rho-dependent transcription termination signal distal to gene IV of f1 encompasses a sequence of ~100 nucleotides in the IG. The endpoints of subclones possessing or lacking terminator function in a heterologous plasmid system are shown in Figures 6.1b and 6.6, but the exact 5' and 3' boundaries of the terminator sequence have not yet been defined. f1 RNA 3'ends formed in vivo at two distinct sites defined by S1 nuclease mapping are shown by large open arrows in Figure 6.6. The 3'end at position 5565 is rho-dependent, while the 3'end at 5650 is rho-independent. In a rho$^-$ host 3'end 5565 is absent, while the amount of 3'end 5650 is unchanged. However, in the rho$^-$ host a 10-20 fold excess of RNA is observed which extends ~200 nucleotides into the downstream part of the IG, and ends at the sites shown by solid arrows in Figure 6.6 near the phage origin of DNA replication.

I do not know whether any of the RNA 3'ends I detect in vivo are generated by a termination event directly, or by subsequent RNA processing events in vivo.

Since S1 nuclease can "nibble" the ends of RNA/DNA hybrids (Shenk et al., 1975), the presence of bands at three adjacent nucleotide positions around the presumed termination sites (Figure 6.4, lanes g,g') may reflect the nibbling activity of S1 under the conditions of the experiment (Grosschedl and Birnstiel, 1980; Green and Roeder, 1980; Aiba et al., 1981). Alternatively, there may be heterogeneity at the actual RNA 3'ends in vivo. None of the RNA 3'ends defined in the experiments described here occur within A-T rich sequences, suggesting that the bands observed are not due to artifactual cleavages of weakly base-paired RNA/DNA hybrids (Hansen et al., 1981). The absence of the 3'end at f1 position ~5565 when RNA from rho$^-$ hosts, or RNA transcribed in vitro in the absence of rho protein, is examined by this technique further suggests that this band is not produced by artifactual S1 cleavage of

the RNA/DNA hybrid.

When the termination site at 5565 is read through in a rho$^-$ host, the amount of "readthrough RNA" observed greatly exceeds the amount of "terminated RNA" detected in a rho$^+$ host by Northern hybridization or S1 nuclease mapping (Figure 6.3, lanes d-g, Figure 6.4, lanes e,g,e',g'; Figure 6.5, lanes b,d). Possible explanations for this phenomenon are: 1) More transcripts coming from upstream promoters pass through the gene IV region of f1 in rho$^-$ hosts. This could be due to readthrough of other rho-dependent termination sites. 2) "Terminated RNA" is less stable than "readthrough RNA." Differential stability of two λ int mRNAs has been shown to depend upon the action of E. coli RNAse III at a site near the 3'end of the longer mRNA (Guaneros et al., 1982; Rosenberg and Schmeissner, 1982). For the f1 RNAs described here, the shorter "terminated RNA" would be presumed less stable and subject to more rapid degradation by E. coli nucleases. However, RNAse III appears not to be involved in this process.

The f1 rho-dependent terminator is located between the IF and F regions of the genome and normally prevents transcription from proceeding through the origin of DNA replication, although termination is not 100% efficient (Figures 6.3, 6.4, 6.5). However, a 10-20 fold increase in transcription into this region is not lethal to the phage in rho$^-$ cells. Transcripts which normally terminate ~68 nucleotides distal to gene IV are extended by ~200 nucleotides in rho$^-$ cells, and terminate at the sites marked with solid arrows within origin hairpins C, D, and E of Figure 6.6. The transcripts do not continue into gene II, and no RNAs longer than ~4200 nucleotides are seen. The high degree of potential secondary structure in the origin region (Schaller, 1978) may facilitate rho-independent termination (Adhya et al., 1979). In contrast, the heterologous terminator plasmid transcripts pass through downstream plasmid sequences into the galK gene in rho$^-$ cells.

The most obvious feature of the terminator sequence is the large hairpin structure which can be drawn distal to gene IV (hairpin A in Figure 6.6). The rho-dependent RNA 3'end occurs within the sequence

CCCT at position 5565 on the distal side of this structure. The RNA 3'end observed at position 5650 also occurs within the sequence CCCT, near the top of hairpin B. The "readthrough RNA" 3'ends in hairpin C around position 5733 also occur in and near the sequence CCCT. However, CCCT also occurs at positions 5493, 5591, and 5753 where no RNA 3'ends have been mapped. Rho protein has been shown to possess a poly(C)-dependent ATPase activity related to its function (Richardson et al., 1975), and it has been suggested that weakly paired cytosine residues are important for rho action (Lowery and Richardson, 1977; Adhya et al., 1979). The sequence (CA)ATCAA common to the λt_{R1}, λt_{RO}, and E. coli $tRNA^{Tyr}$ rho-dependent terminators (Rosenberg et al., 1978; Calva and Burgess, 1980; Küpper et al., 1978) is not present in the f1 IG sequence, although ATCAA does occur within the 5'end of the gene II mRNA (Beck and Zink, 1981). The f1 sequence around the rho-dependent 3'end at 5565 is G-C rich, in contrast to the A-T rich sequences found at previously characterized rho-dependent terminators. The identification of particular sequences within the f1 rho-dependent terminator region required for terminator function awaits further analysis, along with the possible involvement of other E. coli termination factors such as the nusB gene product (Ward and Gottesman, 1981).

References

1. Adhya, S. and Gottesman, M. (1978). Control of transcription termination. Ann. Rev. Biochem. 47: 967-996.

2. Adhya, S., Sarkar, P., Valenzuela, D. and Maitra, U. (1979). Termination of transcription by Escherichia coli RNA polymerase: Influence of secondary structure of RNA transcripts on *ρ*-independent and *ρ*-dependent termination. Proc. Natl. Acad. Sci. USA 76: 1613-1617.

3. Aiba, H., Adhya, S. and de Crombrugghe, B. (1981). Evidence for two functional gal promoters in intact Escherichia coli cells. J. Biol. Chem. 256: 11905-11910.

4. Alberts, B. and Frey, L. (1972). Isolation and characterization of gene 5 protein of filamentous bacterial viruses. J. Mol. Biol. 68: 139-152.

5. Alwine, C. A., Kemp, D. J. and Stark, G. R. (1977). Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc. Natl. Acad. Sci. USA 74: 5350-5354.

6. Beaudoin, J. (1970). Studies on coat protein mutants and on multiple-length particles of coliphage M13. Ph.D. Thesis, University of Wisconsin, Madison.

7. Beck, E., Sommer, E.A., Auerswald, E.A., Kurz, Ch., Zink, B., Osterburg, G., Schaller, H., Sugimoto, K., Sugisaki, H., Okamoto, T. and Takanami, M. (1978). Nucleotide sequence of bacteriophage fd DNA. Nucl.Acids Res. 5: 4495-4503.

8. Beck, E. and Zink, B. (1981). Nucleotide sequence and genome organization of filamentous bacteriophages f1 and fd. Gene 16: 35-58.

9. Betlach, M., Herschfield, V., Chow, L., Brown, W., Goodman, H. M. and Boyer, H. W. (1976). A restriction endonuclease analysis of the bacterial plasmid controlling the EcoRI restriction and modification of DNA. Fed. Proc., Am. Soc. Exp. Biol., 35: 2037-2043.

10. Boeke, J. D. (1981). One and two codon insertion mutants of bacteriophage f1. Mol. Gen. Genet. 181: 288-291.

11. Boeke, J. D. (1982). Filamentous phage coat protein genes: mutagenic alteration of two model membrane proteins. Ph.D. Thesis, The Rockefeller University, New York, New York.

12. Boeke, J. D. and Model, P. (1979). Molecular basis of the am8H1 lesion in bacteriophage M13. Virology 96: 299-301.

13. Boeke, J. D., Model, P. and Zinder, N. D. (1982). Effects of bacteriophage f1 gene III protein on the host cell membrane. Mol. Gen. Genet. 186: 185-192.

14. Boeke, J.D., Russel, M. and Model, P. (1980). Processing of filamentous phage pre-coat protein. Effect of sequence variations near the signal peptidase cleavage site. J. Mol. Biol. 144: 103-116.

15. Boeke, J. D., Vovis, G. F. and Zinder, N. D. (1979). Insertion mutant of bacteriophage f1 sensitive to EcoRI. Proc. Natl. Acad. Sci. USA 76: 2699-2702.

16. Bolivar, F., Rodriguez, R. L., Green, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crosa, J. H. and Falkow, S. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2: 95-113.

17. Branch, A. D., Robertson, H. D. and Dickson,E. (1981). Longer-than-unit length viroid minus strands are present in RNA from infected plants. Proc. Natl. Acad. Sci. USA 78: 6381-6385.

18. Braunitzer, G., Braig, S., Krug, F. and Hobom, G. (1970). Mutanten des Hüllproteins (B-Protein) des Bakteriophagen fd. FEBS Lett. 7: 83-85.

19. Brown, N. L. and Smith, M. (1977). Cleavage specificity of the restriction endonuclease isolated from Haemophilus gallinarum (HgaI). Proc. Natl. Acad. Sci. USA 74: 3213-3216.

20. Calva, E. and Burgess, R. R. (1980). Characterization of a ρ-dependent termination site within the cro gene of bacteriophage lambda. J. Biol. Chem. 255: 11017-11022.

21. Caro, L. G. and Schnös, M. (1966). The attachment of the male-specific bacteriophage f1 to sensitive strains of Escherichia coli. Proc. Natl. Acad. Sci. USA 56: 126-132.

22. Cashman, J. S. and Webster, R. E. (1979). Bacteriophage f1 infection of Escherichia coli: Identification and possible processing of f1-specific mRNAs in vivo. Proc. Natl. Acad. Sci. USA 76: 1169-1173.

23. Cashman, J. S., Webster, R.E. and Steege, D.A. (1980). Transcription of bacteriophage f1. The major in vivo RNAs. J. Biol. Chem. 255: 2554-2562.

24. Chamberlain, J. P. (1979). Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. Anal. Biochem. 98: 132-135.

25. Chan, T.-S., Model, P. and Zinder, N. D. (1975). In vitro protein synthesis directed by separated transcripts of bacteriophage f1 DNA. J. Mol. Biol. 99: 369-382.

26. Chang, C. N., Blobel, G. and Model, P. (1978). Detection of prokaryotic signal peptidase in an Escherichia coli membrane fraction. Endoproteolytic cleavage of nascent f1 pre-coat protein. Proc. Natl. Acad. Sci. USA 75: 361-365.

27. Court, D., Brady, C., Rosenberg, M., Wulff, D. L., Behr, M., Mahoney, M. and Izumi, S. (1980). Control of transcription termination: a rho-dependent termination site in bacteriophage lambda. J. Mol. Biol. 138: 231-254.

28. Davis, R. W., Botstein, D. and Roth, J. R. (1980). Advanced Bacterial Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

29. Denhardt, D. T., Dressler, D. and Ray, D. S. (eds.) (1978). The Single Stranded DNA Phages. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

30. Dotto, G. P., Enea, V. and Zinder, N. D. (1981a). Gene II of phage f1: its functions and its products. Proc. Natl. Acad. Sci. USA 78: 5421-5424.

31. Dotto, G. P., Enea, V. and Zinder, N. D. (1981b). Functional analysis of bacteriophage f1 intergenic region. Virology 114: 463-473.

32. Dunn, J. J. and Studier, F. W. (1981). Nucleotide sequence from the genetic left end of bacteriophage T7 DNA to the beginning of gene 4. J. Mol. Biol. 148: 303-330.

33. Edens, L., Konings, R. N. H. and Schoenmakers, J. G. G. (1975). Physical mapping of the central terminator for transcription on the bacteriophage M13 genome. Nucl. Acids Res. 2: 1811-1820.

34. Edens, L., Konings, R. N. H. and Schoenmakers, J. G. G. (1978). A cascade mechanism of transcription in bacteriophage M13 DNA. Virology 86: 354-367.

35. Edens, L., van Wezenbeek, P., Konings, R.N. H. and Schoenmakers, J. G. G. (1976). Mapping of promoter sites on the genome of bacteriophage M13. Eur. J. Biochem. 70: 577-587.

36. Enea, V., Horiuchi,K., Turgeon, B. G. and Zinder, N. D. (1977). Physical map of defective interfering particles of bacteriophage f1. J. Mol. Biol. 111: 395-414.

37. Enea, V. and Zinder, N. D. (1975). A deletion mutant of bacteriophage f1 containing no intact cistrons. Virology 68: 105-114.

38. Geider, K., Beck, E. and Schaller, H. (1978). An RNA transcribed from DNA at the origin of phage fd single-strand to replicative form conversion. Proc. Natl. Acad. Sci. USA 75: 645-649.

39. Goldsmith, M. E. and Konigsberg, W. (1977). Adsorption protein of the bacteriophage fd: isolation, molecular properties, and location in the virus. Biochemistry 16: 2686-2694.

40. Grant, R. A., Lin, T. C., Konigsberg, W. N. and Webster, R. E. (1981). Structure of the filamentous bacteriophage f1: location of the A, C, and D minor coat proteins. J. Biol. Chem. 256: 539-546.

41. Green, M. R. and Roeder, R. G. (1980). Definition of a novel promoter for the major adenovirus-associated virus mRNA. Cell 22: 231-242.

42. Greenblatt, J., McLimont, M. and Hanly, S. (1981). Termination of transcription by nusA gene protein of Escherichia coli. Nature 292: 215-220.

43. Griffith, J., Manning, M. and Dunn, K. (1981). Filamentous bacteriophage contract into hollow spherical particles upon exposure to a chloroform-water interface. Cell 23: 747-753.

44. Gross, H. J., Domdey, H., Lossow, C., Jank, P., Raba, M., Alberty, H. and Sänger, H. L. (1978). Nucleotide sequence and secondary structure of potato spindle tuber viroid. Nature 273: 203-208.

45. Grosschedl, R. and Birnstiel, M. L. (1980). Identification of regulatory sequences in the prelude sequences of an H2A histone gene by the study of specific deletion mutants in vivo. Proc. Natl. Acad. Sci. USA 77: 1432-1436.

46. Guaneros, G., Montañez, C.,Hernandez, T. and Court, D. (1982). Posttranscriptional control of bacteriophage λ int gene expression from a site distal to the gene. Proc. Natl. Acad. Sci. USA 79: 238-242.

47. Guarente, L., Lauer, G., Roberts, R. M. and Ptashne, M. (1980). Improved methods for maximizing expression of a cloned gene: a bacterium that synthesizes rabbit β-globin. Cell 20: 543-553.

48. Hansen, U., Tenen, D. G., Livingston, D. M. and Sharp, P. A. (1981). T antigen repression of SV40 early transcription from two promoters. Cell 27: 603-612.

49. Herrmann, R., Neugebauer, K., Zentgraf, H. and Schaller, H. (1978). Transposition of a DNA sequence determining kanamycin resistance into the single-stranded genome of bacteriophage fd. Molec. Gen. Genet. 159: 171-178.

50. Hill, D. F. and Petersen, G.B. (1982). Nucleotide sequence of bacteriophage f1 DNA. J. Virol. 44: 32-46.

51. Horiuchi, K., Vovis, G. F., Enea, V. and Zinder, N.D. (1975). Cleavage map of bacteriophage f1: Location of the Escherichia coli B-specific modification sites. J. Mol. Biol. 95: 147-165.

52. Horiuchi, K. and Zinder, N. D. (1976). Origin and direction of synthesis of bacteriophage f1 DNA. Proc. Natl. Acad. Sci. USA 73: 2341-2345.

53. Imai, M. and Shigesada, K. (1978). Studies on the altered rho factor in nitA mutants of Escherichia coli defective in transcription termination. I. Characterization and quantitative determination

of rho in cell extracts. J. Mol. Biol. 120: 451-466.

54. Inoko, H., Shigesada, K. and Imai, M. (1977). Isolation and characterization of conditional-lethal rho mutants of Escherichia coli. Proc. Natl. Acad. Sci. USA 74: 1162-1166.

55. Konings, R. N. H. and Schoenmakers,J. G. G. (1978). Transcription of the filamentous phage genome. In, The Single-Stranded DNA Phages (Denhardt, D. T., Dressler, D. and Ray, D. S., eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 507-530.

56. Küpper, H. Sekiya, T., Rosenberg, M., Egan, J. and Landy, A. (1978). A ρ-dependent termination site in the gene coding for tyrosine tRNA su_3 of Escherichia coli. Nature 272: 423-428.

57. La Farina, M. and Model, P. (1978). Transcription in bacteriophage f1-infected Escherichia coli. I. Translation of the RNA in vitro. Virology 86: 368-375.

58. La Farina, M. and Model, P. (1983). Transcription in bacteriophage f1-infected Escherichia coli: Messenger populations in the infected cell. J. Mol. Biol. 164: 377-393.

59. LeClerc, J. E. and Istock, N. L. (1982). Specificity of UV mutagenesis in the lac promoter of M13 lac hybrid phage DNA. Nature 297: 596-598.

60. Lim, L. W. and Kennell, D. (1979). Models for decay of Escherichia coli lac messenger RNA and evidence for inactivating cleavages between its messenges. J. Mol. Biol. 135: 369-390.

61. Lin, T.C., Webster, R. E. and Konigsberg, W. (1980). Isolation and characterization of the C and D proteins coded by gene IX and gene VI in the filamentous bacteriophage f1 and fd. J. Biol. Chem. 255: 10331-10337.

62. Loeb, T. (1960). Isolation of a bacteriophage specific for the F^+ and Hfr mating types of Escherichia coli K12. Science 131: 932-933.

63. Lowery, C. and Richardson, J. P. (1977). Characterization of the nucleoside triphosphate phosphohydrolase (ATPase) activity of RNA synthesis termination factor ρ. J. Biol. Chem. 252: 1381-1385.

64. Lyons, L. B. and Zinder, N. D. (1972). The genetic map of the filamentous bacteriophage f1. Virology 49: 45-60.

65. Mandel, M. and Higa, A. (1970). Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. 53: 159-162.

66. Maxam, A. M. and Gilbert, W. (1980). Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. 65: 499-560.

67. Mazur, B. J. and Model, P. (1973). Regulation of coliphage f1 single-stranded DNA synthesis by a DNA-binding protein. J. Mol. Biol. 78: 285-300.

68. Mazur, B. J. and Zinder, N. D. (1975). The role of gene V protein in f1 single-strand synthesis. Virology 68: 490-502.

69. McKenney, K., Shimatake, H., Court, D., Schmeissner, U., Brady, C. and Rosenberg, M. (1981). A system to study promoter and terminator signals recognized by Escherichia coli RNA polymerase. In, Gene Amplification and Analysis, Vol. II: Analysis of Nucleic Acids by Enzymatic Methods. (Chirikjian, J. C. and Papas, T. S., eds.), Elsevier-North Holland, pp. 383-415.

70. McMaster, G. K. and Carmichael, G. C. (1977). Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA 74: 4835-4838.

71. Messing, J., Gronenborn, B., Müller-Hill, B. and Hofschneider, P. H. (1977). Filamentous coliphage M13 as a cloning vehicle: Insertion of a HindII fragment of the lac regulatory region in M13 replicative form in vitro. Proc. Natl. Acad. Sci. USA 74: 3642-3646.

72. Meyer, T. F. and Geider, K. (1979). Bacteriophage fd gene II protein. II. Specific cleavage and relaxation of supercoiled RF from filamentous phages. J. Biol. Chem. 254: 12642-12646.

73. Miller, J. H. (1972). Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

74. Miller, J. H. (1982). Carcinogens induce targeted mutations in Escherichia coli. Cell 31: 5-7.

75. Miller, J. H. and Reznikoff, W. S. (eds.) (1978). The Operon. Cold Spring Harbor Laboratory, Cold Spring Harbor,New York.

76. Model, P. and Zinder, N. D. (1974). In vitro synthesis of bacteriophage f1 proteins. J. Mol. Biol. 83: 231-251.

77. Nakashima, Y. and Konigsberg, W. (1974). Reinvestigation of a region of the fd bacteriophage coat protein sequence. J. Mol. Biol. 88: 598-600.

78. Nelson, F. K., Friedman, S. M. and Smith, G. P. (1981). Filamentous phage DNA cloning vectors: a noninfective mutant with a nonpolar deletion in gene III. Virology 108: 338-350.

79. Okamoto, T., Sugimoto, K., Sugisaki, H. and Takanami, M. (1975). Studies on bacteriophage fd DNA. II. Localization of RNA initiation sites on the cleavage map of the fd genome. J. Mol. Biol. 95: 33-44.

80. Okamoto, T., Sugimoto, K., Sugisaki, H. and Takanami, M. (1977). DNA regions essential for the function of a bacteriophage fd promoter. Nucl. Acids Res. 4: 2213-2222.

81. Okamoto, T., Sugiura, M. and Takanami, M. (1969). Length of RNA transcribed on the replicative form DNA of coliphage fd. J. Mol. Biol. 45: 101-111.

82. Pieczenik, G., Model, P. and Robertson, H. D. (1974). Sequence and symmetry in ribosome binding sites of bacteriophage f1 RNA. J. Mol. Biol. 90: 191-214.

83. Platt, T. (1981). Termination of transcription and its regulation in the tryptophan operon of E. coli. Cell 24: 10-23.

84. Pratt, D. and Erdahl, W. S. (1968). Genetic control of bacteriophage M13 DNA synthesis. J. Mol. Biol. 37: 181-200.

85. Pratt, D., Tzagoloff, H. and Beaudoin, J. (1969). Conditional lethal mutants of the small filamentous coliphage M13. II. Two genes for coat proteins. Virology 39: 42-53.

86. Pratt, D., Tzagoloff, H. and Erdahl, W. S. (1966). Conditional lethal mutants of the small filamentous coliphage M13. I. Isolation, complementation, cell killing, time of cistron action. Virology 30: 397-410.

87. Reznikoff, W. S. and Abelson, J. N. (1978). The lac promoter. In, The Operon (Miller, J. H. and Reznikoff, W. S. eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 221-243.

88. Richardson, J. P., Grimley, C. and Lowery, C. (1975). Transcription termination factor rho activity is altered in Escherichia coli with suA gene mutations. Proc. Natl. Acad. Sci. USA 72: 1725-1728.

89. Rivera, M. J., Smits, M. A., Quint, W., Schoenmakers, J. G. G. and Konings, R. N. H. (1978). Expression of bacteriophage M13 DNA in vivo. Localization of the transcription initiation and termination signal of the mRNA coding for the major capsid protein. Nucl. Acids Res. 5: 2895-2912.

90. Roberts, J. (1969). Termination factor for RNA synthesis. Nature 224: 1168-1174.

91. Rosenberg, M. and Court, D. (1979). Regulatory sequences involved in the promotion and termination of RNA transcription. Ann. Rev. Genet. 13: 319-353.

92. Rosenberg, M., Court, D., Shimatake, H., Brady, C. and Wulff, D. L. (1978). The relationship between function and DNA sequence in an intercistronic regulatory region in phage lambda. Nature 272: 414-423.

93. Rosenberg, M. and Schmeissner, U. (1982). The regulation of gene expression by transcription termination and RNA processing. In, Interaction of Translational and Transcriptional Controls in the Regulation of Gene Expression (Safer, B. and Grunberg-Manago, M., eds.), Elsevier Biomedical, New York, pp. 1-16.

94. Rossomando, E. F. and Zinder, N. D. (1968). Studies on the bacteriophage f1. I. Alkali-induced disassembly of the phage into DNA and protein. J. Mol. Biol. 36: 387-399.

95. Sanger, F. and Coulson, A. R. (1978). The use of thin acrylamide gels for DNA sequencing. FEBS Lett. 87: 107-110.

96. Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5467.

97. Schaller, H. (1978). The intergenic region and the origins for filamentous phage DNA replication. Cold Spring Harbor Symp. Quant. Biol. XLIII: 401-408.

98. Schaller, H., Beck, E. and Takanami, M. (1978). Sequence and regulatory signals of the filamentous phage genome. In, The Single-Stranded DNA Phages (Denhardt, D. T., Dressler, D. and Ray, D. S., eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 139-163.

99. Seeburg, P. H., Nüsslein, C. and Schaller, H. (1977). Interaction of RNA polymerase with promoters from bacteriophage fd. Eur. J. Biochem. 74: 107-113.

100. Seeburg, P. H. and Schaller,H. (1975). Mapping and characterization of promoters in bacteriophage fd, f1, and M13. J. Mol. Biol. 92: 261-277.

101. Shenk, T. E., Rhodes, C., Rigby, P. W. and Berg, P. (1975). Biochemical method for mapping mutational alterations in DNA with S1 nuclease: The location of deletions and temperature-sensitive mutations in simian virus 40. Proc. Natl. Acad. Sci. USA 72: 989-993.

102. Shigesada, K. and Imai, M. (1978). Studies on the altered rho factor in nitA mutants of Escherichia coli defective in transcription termination. II. Purification and molecular properties of the mutant rho. J. Mol. Biol. 120: 467-486.

103. Simons, G. F. M., Konings, R. N. H. and Schoenmakers, J. G. G. (1981). Genes VI, VII, and IX of phage M13 code for minor capsid proteins of the virion. Proc. Natl. Acad. Sci. 78: 4194-4198.

104. Smilowitz, H. (1974). Bacteriophage f1 infection: fate of the parental major coat protein. J. Virol. 13: 94-99.

105. Smits, M. A., Schoenmakers, J. G. G. and Konings, R. N. H. (1980). Expression of bacteriophage M13 DNA in vivo. Isolation, identification, and characterization of phage-specific mRNA species. Eur. J. Biochem. 112: 309-321.

106. Smits, M. A., Simons, G., Konings, R. N. H. and Schoenmakers, J. G. G. (1978). Expression of bacteriophage M13 DNA in vivo. I. Synthesis of phage specific RNA and protein in minicells. Biochim. Biophys. Acta 521: 27-44.

107. Studier, W. (1975). Genetic mapping of a mutation that causes ribonuclease III deficiency in Escherichia coli. J. Bacteriol. 124: 307-316.

108. Suggs, S. V. and Ray, D. S. (1977). Replication of bacteriophage M13. XI. Localization of the origin for M13 single-strand synthesis. J. Mol. Biol. 110: 147-163.

109. Sugimoto, K., Sugisaki, H., Okamoto, T. and Takanami, M. (1977). Studies on bacteriophage fd DNA. IV. The sequence of messenger RNA for the major coat protein gene. J. Mol. Biol. 111: 487-507.

110. Sugisaki, H. (1978). Recognition sequence of a restriction endonuclease from Haemophilus gallinarum. Gene 3: 17-28.

111. Sutcliffe, J. G. (1979). Complete nucleotide sequence of the Escherichia coli plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. XLIII: 77-90.

112. Tabak, H. F., Griffith, J., Geider, K., Schaller, H. and Kornberg, A. (1974). Initiation of deoxyribonucleic acid synthesis. VII. A unique location of the gap in the M13 replicative duplex synthesized in vitro. J. Biol. Chem. 249: 3049-3054.

113. Takanami, M. (1973). Specific cleavage of coliphage fd DNA by five different restriction endonucleases from Haemophilus genus. FEBS Lett. 34: 318-322.

114. Takanami, M., Okamoto, T. and Sugiura, M. (1970). The starting nucleotide sequences and size of RNA transcribed in vitro on phage DNA templates. Cold Spring Harbor Symp. Quant. Biol. XXXV: 179-187.

115. Takanami, M., Okamoto, T. and Sugiura, M. (1971). Termination of RNA transcription on the replicative form DNA of bacteriophage fd. J. Mol. Biol. 62: 81-88.

116. Thomas, P. S. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA 77: 5201-5205.

117. Ullrich, A., Shine, J., Chirgwin, J., Pictet, R., Tischer, E., Rutler, W. and Goodman, H. (1977). Rat insulin genes: Construction of plasmids containing the coding sequences. Science 196: 1313-1319.

118. van den Hondel, C. A., Pennings, L. and Schoenmakers, J. G. G. (1976). Restriction enzyme-cleavage maps of bacteriophage M13: Existence of an intergenic region on the M13 genome. Eur. J. Biochem. 68: 55-70.

119. van Wezenbeek, P. M. G. F., Hulsebos, T. J. M. and Schoenmakers, J. G. G. (1980). Nucleotide sequence of the filamentous bacteriophage M13 genome: comparison with phage fd. Gene 11: 129-148.

120. Visvader, J. E., Gould, A. R., Bruening, G. E. and Symons, R. H. (1982). Citrus exocortis viroid: Nucleotide sequence and secondary structure of an Australian isolate. FEBS Lett. 137: 288-292.

121. Vogel, H. J. and Bonner, D. M. (1956). Acetylornithinase of Escherichia coli: partial purification and some properties. J. Biol. Chem. 218: 97-106.

122. Vovis, G.F., Horiuchi, K. and Zinder, N. D. (1975). Endonuclease EcoRII restriction of bacteriophage f1 DNA in vitro: Ordering of genes V and VII, location of an RNA promoter for gene VIII. J. Virol. 16: 674-684.

123. Ward, D. F. and Gottesman, M. E. (1981). The nus mutations affect transcription termination in Escherichia coli. Nature 292: 212-215.

124. Webster, R. E. and Cashman, J. S. (1978). Morphogenesis of the filamentous single-stranded DNA phages. In, The Single-Stranded DNA Phages (Denhardt, D. T., Dressler, D. and Ray, D. S., eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 557-569.

125. Wickner, W. (1976). Asymmetric orientation of phage M13 coat protein in Escherichia coli cytoplasmic membranes and in synthetic lipid vesicles. Proc. Natl. Acad. Sci. USA 73: 1159-1163.

126. Wu, A. M., Christie, G. and Platt, T. (1981). Tandem termination sites in the tryptophan operon of Escherichia coli. Proc. Natl. Acad. Sci. USA 78: 2913-2917.

127. Yen, T. S. and Webster, R. E. (1981). Bacteriophage f1 gene II and X proteins. Isolation and characterization of the products of two overlapping genes. J. Biol.Chem. 256: 11259-11265.

128. Zinder, N. D., Valentine, R. C., Roger, M. and Stoeckenius, W. (1963). f1, a rod-shaped male-specific bacteriophage that contains DNA. Virology 20: 638-640.

End